

**PREVENTION AND TREATMENT OF CARDIOVASCULAR PATHOLOGIES WITH TAMOXIFEN ANALOGUES****Field of the Invention**

5        This invention relates generally to the prevention and treatment of cardiovascular pathologies. More specifically, a method for treating or preventing atherosclerosis is provided.

**Background of the Invention**

10        Many pathological conditions have been found to be associated with smooth muscle cell proliferation. Such conditions include restenosis, atherosclerosis, coronary heart disease, thrombosis, myocardial infarction, stroke, smooth muscle neoplasms such as leiomyoma and leiomyosarcoma of the bowel and uterus, uterine fibroid or fibroma, and obliterative disease of vascular grafts and transplanted organs. The mechanisms of abnormal smooth muscle  
15        cell proliferation are not yet well understood.

For example, percutaneous transluminal coronary angioplasty (PTCA) is widely used as the primary treatment modality in many patients with coronary artery disease. PTCA can relieve myocardial ischemia in patients with coronary artery disease by reducing lumen obstruction and improving coronary flow. The  
20        use of this surgical procedure has grown rapidly, with 39,000 procedures performed in 1983, nearly 150,000 in 1987, 200,000 in 1988, 250,000 in 1989, and over 500,000 PTCA's per year are estimated by 1994. Stenosis following PTCA remains a significant problem, with from 25% to 35% of the patients developing restenosis within 1 to 3 months. Restenosis results in significant  
25        morbidity and mortality and frequently necessitates further interventions such as repeat angioplasty or coronary bypass surgery. No surgical intervention or post-surgical treatment (to date) has proven effective in preventing restenosis.

The processes responsible for stenosis after PTCA are not completely understood but may result from a complex interplay among several different  
30        biologic agents and pathways. Viewed in histological sections, restenotic lesions may have an overgrowth of smooth muscle cells in the intimal layers of the vessel. Several possible mechanisms for smooth muscle cell proliferation after

PTCA have been suggested. For example, Barath et al. (U. S. Patent No. 5,242,397) disclose delivering cytotoxic doses of protein kinase C inhibitors, including tamoxifen, locally by catheter to the site of the atherosclerotic lesion.

- Compounds that reportedly suppress smooth muscle proliferation *in vitro* may have undesirable pharmacological side effects when used *in vivo*. Heparin is an example of one such compound, which reportedly inhibits smooth muscle cell proliferation *in vitro* but when used *in vivo* has the potential adverse side effect of inhibiting coagulation. Low molecular weight fragments of heparin, while having reduced anti-coagulant activity, have the undesirable pharmacological property of a short pharmacological half-life. Attempts have been made to solve such problems by using a double balloon catheter, i.e., for regional delivery of the therapeutic agent at the angioplasty site (e.g., U.S. Pat. No. 4,824,436), and by using biodegradable materials impregnated with a drug, i.e., to compensate for problems of short half-life (e.g., U.S. Pat. No. 4,929,602).

- In general, atherosclerosis is a cardiovascular disease in which the vessel wall is remodeled, compromising the lumen of the vessel. The atherosclerotic remodeling process involves accumulation of cells, both smooth muscle cells and monocyte/macrophage inflammatory cells, in the intima of the vessel wall. These cells take up lipid to form a mature atherosclerotic lesion. Although the formation of these lesions is a chronic process, occurring over decades of an adult human life, the majority of the morbidity associated with atherosclerosis occurs when a lesion ruptures, releasing thrombogenic debris that rapidly occludes the artery. When such an acute event occurs in the coronary artery, myocardial infarction can ensue, and in the worst case, can result in death.

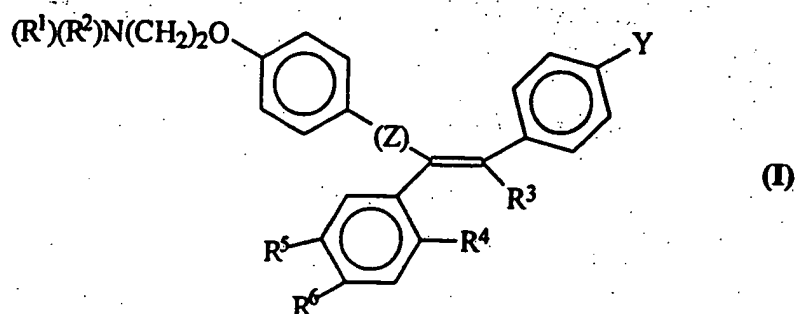
- The formation of the atherosclerotic lesion can be considered to occur in five overlapping stages such as migration, lipid accumulation, recruitment of inflammatory cells, proliferation of vascular smooth muscle cells, and extracellular matrix deposition. Each of these processes can be shown to occur in man and in animal models of atherosclerosis, but the relative contribution of each to the pathology and clinical significance of the lesion is unclear.

Thus, a need exists for therapeutic methods and agents to treat cardiovascular pathologies, such as atherosclerosis and other conditions related to coronary artery disease.

### Summary of the Invention

- 5 A therapeutic method for preventing or treating a cardiovascular or vascular indication characterized by a decreased lumen diameter is provided. The method comprises administering to a mammal at risk of, or afflicted with, said cardiovascular indication, a cytostatic dose of a therapeutic agent that elevates the level of TGF-beta, such as a compound of formula (I)

10



- wherein Z is C=O or a covalent bond; Y is H or O(C<sub>1</sub>-C<sub>4</sub>)alkyl, R<sup>1</sup> and R<sup>2</sup> are individually (C<sub>1</sub>-C<sub>4</sub>)alkyl or together with N are a saturated heterocyclic group, R<sup>3</sup> is ethyl or chloroethyl, R<sup>4</sup> is H, R<sup>5</sup> is I, O(C<sub>1</sub>-C<sub>4</sub>)alkyl or H and R<sup>6</sup> is I, O(C<sub>1</sub>-C<sub>4</sub>)alkyl or H with the proviso that when R<sup>4</sup>, R<sup>5</sup>, and R<sup>6</sup> are H, R<sup>3</sup> is not ethyl; or a pharmaceutically acceptable salt, including mixtures thereof. The cytostatic dose is effective to directly or indirectly increase the level of TGF-beta in a mammal afflicted with said indication. Preferably, the effective amount inhibits smooth muscle cell proliferation, inhibits lipid accumulation, increases plaque stability, or any combination thereof. Thus, in this embodiment of the invention, the compound of formula (I) does not include tamoxifen, raloxifene or droloxifene. However, in other embodiments of the invention, the compound of

formula(I) can include the following:  $R^4$  together with  $R^3$  is  $-CH_2-CH_2-$  or  $-S-$ ,  $R^5$  is OH, or  $R^4$ ,  $R^5$ , and  $R^6$  are H and  $R^3$  is ethyl.

A therapeutic method is provided for treating or preventing cardiovascular pathologies, such as conditions selected from the group consisting of atherosclerosis, thrombosis, myocardial infarction, and stroke. The method comprises the systemic or local administration of an amount of compound of formula (I). The amount is effective to increase the level of TGF-beta in said mammal afflicted with one of these conditions.

The administered compound of formula (I) can act on vascular smooth muscle cells (VSMC) to inhibit the pathological activity of these smooth muscle cells, can inhibit the activation of endothelial cells, can inhibit lipid accumulation by vessels, decrease lesion formation or development, and can increase plaque stability. Preferably, the compound significantly reduces the rate of completion of the cell cycle and cell division, and preferably is administered at cytostatic, as opposed to cytotoxic, doses. A preferred embodiment of the invention comprises treatment of atherosclerosis, wherein the compound of formula (I), such as idoxifene or idoxifene salt, inhibits lipid accumulation by vascular smooth muscle cells and/or stabilizes an arterial lesion associated with atherosclerosis, i.e., increases plaque stability, to prevent rupture or growth of the lesion. As exemplified hereinbelow, orally administered tamoxifen significantly inhibits the formation of lipid lesions, induced by a high fat diet, in C57B16 mice and in the transgenic apo(a) mouse. The 90% reduction in lesion area and number in both of these mouse models indicates that tamoxifen affects the accumulation of lipid in the cells and stroma of the vessel wall. The inhibition of lipid accumulation and lesion development in these treated mice indicates that tamoxifen and analogs thereof, as well as compounds of formula (I), may inhibit the development of atherosclerotic lesions in humans by inhibiting lipid accumulation, in addition to decreasing smooth muscle cell proliferation.

Other preferred embodiments of the invention comprise the local administration of the compound of formula (I) to an arterial lesion associated with atherosclerosis, and a kit to accomplish said administration.

A further embodiment of the invention is a method for preventing cardiovascular pathologies in a mammal at risk of such a condition. Such conditions include atherosclerosis, thrombosis, myocardial infarction, and stroke. The method comprises the administration of an amount of the compound of formula (I) to a mammal, such as a human, effective to increase the level of TGF-beta in said mammal. The amount of the compound is administered over time as a preventative measure. Preferably, the compound is administered orally, in a series of spaced doses.

A further embodiment of the invention is a method for inhibiting smooth muscle cell (SMC) proliferation associated with procedural vascular trauma as by the systemic or localized catheter or non-catheter administration to a mammal, such as a human patient, subjected to said procedure, an effective cytostatic SMC proliferation inhibitory amount of a compound of formula (I), or a pharmaceutically acceptable salt thereof. The systemic administration can be accomplished by oral or parenteral administration of one of more suitable unit dosage forms, which, as discussed below, may be formulated for sustained release. The administration of the agents of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses, either before, during, or after the procedural vascular trauma, before and during, before and after, during and after, or before, during and after the procedural trauma.

As used herein, the term "procedural vascular trauma" includes the effects of surgical/mechanical interventions into mammalian vasculature, but does not include vascular trauma due to the organic vascular pathologies listed hereinabove.

Thus, procedural vascular traumas within the scope of the present treatment method include (1) organ transplantation, such as heart, kidney, liver and the like, e.g., involving vessel anastomosis; (2) vascular surgery, such as

coronary bypass surgery, biopsy, heart valve replacement, atherectomy, thrombectomy, and the like; (3) transcatheter vascular therapies (TVT) including angioplasty, e.g., laser angioplasty and PTCA procedures discussed hereinbelow, employing balloon catheters, and indwelling catheters; (4) vascular grafting using natural or synthetic materials, such as in saphenous vein coronary bypass grafts, dacron and venous grafts used for peripheral arterial reconstruction, etc.; (5) placement of a mechanical shunt, such as a PTFE hemodialysis shunt used for arteriovenous communications; and (6) placement of an intravascular stent, which may be metallic, plastic or a biodegradable polymer. See U.S. patent application Serial No. 08/389,712, filed February 15, 1995, which is incorporated by reference herein. For a general discussion of implantable devices and biomaterials from which they can be formed, see H. Kambic et al., "Biomaterials in Artificial Organs", Chem. Eng. News, 30 (April 14, 1986), the disclosure of which is incorporated by reference herein.

15 In the case of organ transplantation, the entire organ, or a portion thereof, may be infused with a solution of the compound of formula (I), prior to implantation. Likewise, in vascular surgery, the termini of the vessels subject to anastomosis can be infused with the compound of formula (I), or the antiproliferative agents can be delivered from pretreated sutures or staples.

20 The delivery of an agent that elevates the level of TGF-beta, e.g., TGF-beta activators or production stimulators, to the lumen of a vessel via catheter, before, during or after angioplasty, is discussed in detail below. A stent or shunt useful in the present method can comprise a biodegradable coating or porous non-biodegradable coating, having dispersed therein the sustained-release dosage form. In the alternative embodiment, a biodegradable stent or shunt may also have the therapeutic agent impregnated therein, i.e., in the stent or shunt matrix. Utilization of a biodegradable stent or shunt with the therapeutic agent impregnated therein is further coated with a biodegradable coating or with a porous non-biodegradable coating having the sustained release-dosage form dispersed therein is also contemplated. This embodiment of the invention would provide a differential release rate of the therapeutic agent, i.e., there would be a

faster release of the therapeutic agent from the coating followed by delayed release of the therapeutic agent that was impregnated in the stent or shunt matrix upon degradation of the stent or shunt matrix. The intravascular stent or shunt thus provides a mechanical means of maintaining or providing an increase in luminal area of a vessel, and the antiproliferative agent inhibits the VSMC proliferative response induced by the stent or shunt, which can cause occlusion of blood flow and coronary failure.

For local administration during grafting, the *ex vivo* infusion of the antiproliferative agent into the excised vessels (arteries or veins) to be used for vascular grafts can be accomplished. In this aspect of the invention, the vessel that is to serve as the graft is excised or isolated and subsequently distended by an infusion of a solution of the therapeutic agent, preferably by pressure infusion. Of course, grafts of synthetic fiber can be precoated with TMX and/or compounds of formula (I) prior to *in vivo* placement.

A further aspect of the invention is a method comprising inhibiting vascular smooth muscle cell proliferation associated with procedural vascular trauma due to organ transplantation, vascular surgery, angioplasty, shunt placement, stent placement or vascular grafting comprising administration to a mammal, such as a human, subjected to said procedural trauma an effective antiproliferative amount of a compound of formula (I) or a pharmaceutically acceptable salt thereof. Administration may be systemic, as by oral or parenteral administration, or local, as to the site of the vascular trauma, or both.

Yet a further aspect of the invention provides a method comprising inhibiting non-aortal vascular smooth muscle cell proliferation associated with procedural vascular trauma comprising administering an effective cytostatic antiproliferative amount of tamoxifen, a structural analog thereof, a compound of formula (I) which includes when  $R^4$  together with  $R^3$  is  $-CH_2-CH_2-$  or  $-S-$ , or  $R^5$  is OH, including the pharmaceutically acceptable salts thereof, to a mammal, such as a human, subjected to said procedural vascular trauma. Said administration can be systemic or by local, catheter or non-catheter delivery to

the site of the trauma. A preferred embodiment of the invention comprises inhibiting non-aortal vascular smooth muscle cells in a non-coronary artery.

Also provided is a kit comprising packing material enclosing, separately packaged, a catheter, a stent, a shunt or a synthetic graft and a unit dosage form of an amount of a compound of formula (I) and/or tamoxifen effective to accomplish these therapeutic results when delivered locally, as well as instruction means for its use, in accord with the present methods.

Another embodiment of the present invention is a method for identifying an agent which increases the level of TGF-beta, e.g., the agent is a TGF-beta activator or production stimulator. Human vascular smooth muscle cells (hVSMC) are cultured with an amount of the agent effective to reduce or inhibit the rate of hVSMC proliferation. The hVSMC are then contacted with an amount of a moiety which specifically binds to TGF-beta in an amount effective to block the binding of TGF-beta to the TGF-beta receptors of said hVSMC and then the rate of proliferation is determined. The method can also include the culture of rat aortic vascular smooth muscle cells (rVSMC) with an amount of the same agent effective to reduce or inhibit the rate of proliferation of rVSMC. The rVSMC are then contacted with an amount of a moiety which specifically binds to TGF-beta in an amount effective to block the binding of TGF-beta to the TGF-beta receptors of said hVSMC and then the rate of proliferation is determined. The rate of proliferation in treated rVSMC and treated hVSMC relative to untreated rVSMC and hVSMC, respectively, after contact with the moiety indicates that the reduction of proliferation is due to an increase in the level of TGF-beta in rVSMC and hVSMC by said agent, and suggests that rVSMC and hVSMC would be amenable to treatment by the administration of said agent *in vivo*.

Agents useful in the practice of the invention include agents that elevate or increase the level of TGF-beta, e.g., TGF-beta activators and TGF-beta production stimulators, compounds of formula (I) which include when R<sup>4</sup> together with R<sup>3</sup> is -CH<sub>2</sub>-CH<sub>2</sub>- or -S-, or R<sup>3</sup> is OH, tamoxifen, and structural analogs of tamoxifen. These agents and compounds, including their salts and



mixtures thereof, may be employed in the practice of the present invention to prevent or treat other conditions characterized by inappropriate or pathological activity of vascular smooth muscle cells or endothelial cells, excluding the inappropriate proliferation or pathological activity of neoplastic vascular smooth muscle cells or neoplastic endothelial cells. Thus, it is envisioned that the methods of the present invention preferably do not include the treatment of neoplastic vascular tissue.

The agents of the invention, which increase the level of TGF-beta, inhibit abnormal activity of vascular smooth muscle cells and endothelial cells.

Preferred agents of the invention include compounds of formula (I). Preferred compounds of formula (I) include those wherein Z is a covalent bond, Y is H, R<sup>3</sup> is ClCH<sub>2</sub>CH<sub>2</sub>, R<sup>5</sup> or R<sup>6</sup> is iodo, R<sup>4</sup> is H, R<sup>1</sup> and R<sup>2</sup> are each CH<sub>3</sub> or together with N are pyrrolidino, hexamethyleneimino or piperidino. These agents or compounds can include structural analogs of tamoxifen (including derivatives of TMX and derivatives of said analogs) having equivalent bioactivity. Such analogs include idoxifene (IDX)(E-1-[4-[2-N-pyrrolidino]ethoxy]phenyl)-1-(4-iodophenyl)-2-phenyl-1-butene), raloxifene, 3-iodotamoxifen, 4-iodotamoxifen, droloxifene, tomremifene, and the pharmaceutically acceptable salts thereof.

Also provided are a method and a kit to determine the presence and amount of TGF-beta in a sample containing TGF-beta. The method for the determination of TGF-beta *in vitro* can be used to identify a patient at risk for atherosclerosis and/or monitor a recipient that has received one or more administrations of a therapeutic agent which increases the level of TGF-beta, or to monitor active TGF-beta levels in an individual. Blood serum or plasma from an individual, patient or recipient is contacted with a capture moiety to form a capture complex of said capture moiety and TGF-beta. Preferably, the capture moiety is an immobilized capture moiety. The capture complex is then contacted with a detection moiety capable of binding TGF-beta comprising a detectable label. A binding site for a detectable label, to form a detectable complex. The presence and amount, or absence, of the detectable complex is then determined.

thereby determining the presence and amount, or absence, of TGF-beta in the blood of the patient or recipient.

A test kit for determining TGF-beta *in vitro* includes packaging material enclosing (a) a capture moiety capable of binding TGF-beta, and (b) a detection moiety capable of binding to TGF-beta, where the detection moiety has a detectable label or a binding site for a detectable label. The capture moiety and the detection moiety are separately packaged in the test kit. Preferably, the capture moiety is solid substrate-immobilized. Preferably, the capture moiety is the TGF-beta type II receptor extracellular domain. More preferably, the TGF-beta type II receptor extracellular domain is derived from a bacterial expression system. The kit can also comprise instruction means for correlation of the detection or determination of TGF-beta with the identification of the patients or monitoring discussed above.

Further provided is a method for upregulating cellular mRNA coding for TGF-beta. Cells (e.g., smooth muscle cells) amenable to such manipulation of mRNA accumulation are identified in the manner described herein and are exposed to an effective amount of a TGF-beta mRNA regulator (i.e., a subset of TGF-beta production stimulators), either free or in a sustained-release dosage form. In this manner, TGF-beta production is stimulated.

In addition, methods for using TGF-beta to maintain and increase vessel lumen diameter in a diseased or injured mammalian vessel are described.

Also provided is a therapeutic method of increasing the level of TGF-beta in a mammal in need thereof. The method comprises the administration of an effective amount of a compound of formula (I), which includes when R<sup>4</sup> together with R<sup>3</sup> is -CH<sub>2</sub>-CH<sub>2</sub>- or -S-, R<sup>5</sup> is OH, or R<sup>4</sup>, R<sup>5</sup>, and R<sup>6</sup> are H and R<sup>3</sup> is ethyl. A preferred embodiment of the invention is a mammal that is diabetic. Diabetics suffer from a plethora of indications, one of which is a decrease in the level of TGF-beta, as described hereinbelow.

Diabetics are prone to vascular disease. Vascular disease includes, but is not limited to, myocardial infarction, atherosclerosis, arteriosclerosis, and small vessel disease. Moreover, the leading causes of death in diabetics are myocardial

infarction and atherosclerosis. Thus, the present invention further provides a method to treat diabetics at risk of, or afflicted with, vascular disease. The method comprises the administration of an effective amount of an agent that elevates the level of TGF-beta, such as a compound of formula (I) which includes when R<sup>4</sup> together with R<sup>3</sup> is -CH<sub>2</sub>-CH<sub>2</sub>- or -S-, or R<sup>5</sup> is OH, tamoxifen or a structural analog thereof. The amount is effective to directly or indirectly increase the level of TGF-beta in said diabetic. The amount administered is preferably effective to inhibit the proliferation of vascular tissue. A preferred embodiment of the invention includes the administration of idoxifene, 3-iodotamoxifen, 4-iodotamoxifen, raloxifene, droloxifene, toremifene, or a pharmaceutically acceptable salt thereof.

#### Description of the Drawings

Figures 1 and 2 depict pathways for the modulation of vascular smooth muscle cell proliferation *in vivo*.

Figure 3A depicts the reduction in TGF-beta binding to the TGF-beta receptor (R2X) in the presence of increasing amounts of lipoprotein.

Figure 3B depicts the amount of TGF-beta necessary to half maximally inhibit mink lung cell proliferation in the presence of increasing amounts of lipoprotein.

Figure 4 depicts the association of TGF-beta with different lipoprotein classes. Profile of lipoprotein particle elution measured as total cholesterol (.....) and TGF-beta elution (open circles) following separation of the lipoprotein fraction ( $d < 1.215 \text{ g/cm}^3$ ) by gel filtration chromatography. The position of the major lipoprotein classes are marked by reference to the elution times of the major apolipoproteins. (a) Healthy individual A (b) Healthy individual C (c) Diabetic individual K (d) Diabetic individual L. Letters designating the individuals shown refer to individuals in Table 8.

#### Detailed Description of the Invention

As used herein the following terms have the meanings as set forth below:

"Proliferation," means an increase in cell number, i.e., by mitosis of the cells.

"Abnormal or Pathological or Inappropriate Activity or Proliferation" means division, growth or migration of cells occurring more rapidly or to a significantly greater extent than typically occurs in a normally functioning cell of the same type, or in lesions not found in healthy tissues.

"Expressed" means mRNA transcription and translation with resultant synthesis, glycosylation, and/or secretion of a polypeptide by a cell, e.g., chondroitin sulfate proteoglycan (CSPG) synthesized by a vascular smooth muscle cell or pericyte.

"Vascular disease" includes, but is not limited to, myocardial infarction, atherosclerosis, arteriosclerosis, and small vessel disease. Small vessel disease includes, but is not limited to, silent myocardial infarction, vascular insufficiency in the limbs, peripheral neuropathy and retinopathy.

"Vascular tissue," as used herein, includes non-neoplastic smooth muscle cells and non-neoplastic endothelial cells.

The term "tamoxifen", as used herein, includes trans-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine, and the pharmaceutically acceptable salts thereof, which are capable of enhancing the production or activation of TGF-beta. The activated form of TGF-beta, in turn, inhibits endothelial cell and vascular smooth muscle cell activity. Isomers and derivatives of the aforementioned chemical compound are also included within the scope of the term "tamoxifen" for the purposes of this disclosure.

The term "structural analogs thereof" with respect to tamoxifen includes, but is not limited to, all of the compounds of formula (I) which are capable of enhancing production or activation of TGF-beta. See, for example, U.S. Patent Nos. 4,536,516, 5,457,113, 5,047,431, 5,441,986, 5,426,123, 5,384,332, 5,453,442, 5,492,922, 5,462,937, 5,492,926, 5,254,594, and U.K. Patent 1,064,629.

Because tamoxifen (TMX) causes liver carcinogenicity in rats and has been correlated with an increased risk of endometrial cancer in women and may

increase the risk of certain gut cancers, other tamoxifen analogs may be considered safer to administer if they are less carcinogenic. The carcinogenicity of TMX has been attributed to the formation of covalent DNA adducts. Of the TMX analogs and derivatives, only TMX and toremifene have been studied for long-term carcinogenicity in rats and these studies provide strong evidence that covalent DNA adducts are involved in rodent hepatocarcinogenicity of TMX. Toremifene, which exhibits only a very low level of hepatic DNA adducts, was found to be non-carcinogenic. See Potter et al., Carcinogenesis, **15**, 439 (1994). A preferred embodiment of the invention includes the use of a compound of formula (I) which includes when R<sup>4</sup> together with R<sup>3</sup> is -CH<sub>2</sub>-CH<sub>2</sub>- or -S-, or R<sup>5</sup> is OH, that forms DNA adducts at a reduced level relative to TMX. A more preferred embodiment of the invention includes a compound of formula (I) which includes when R<sup>4</sup> together with R<sup>3</sup> is -CH<sub>2</sub>-CH<sub>2</sub>- or -S-, or R<sup>5</sup> is OH, that does not form DNA adducts. The extent of DNA adduct formation by an agent or a compound is determined by methods well known to the art.

It is postulated that 4-hydroxylation of TMX yields electrophilic alkylating agents which alkylate DNA through the ethyl group of TMX. This mechanistic hypothesis explains the low level of DNA adduct formation by the non-TMX analogs of formula (I), including the TMX analog toremifene and the absence of DNA adducts detected for the analogs 4-iodotamoxifen and idoxifene. Thus, all of these analogs are likely to be free from the risk of carcinogenesis in long term use. See Potter et al., *supra*. Idoxifene includes (E)-1-[4-[2-(N-pyrrolidino)ethoxy]phenyl]-1-(4-iodophenyl)-2-phenyl-1-butene and its pharmaceutically acceptable salts and derivatives. See R. McCague et al., Organic Preparations and Procedures Int., **26**, 343 (1994) and S.K. Chandler et al., Cancer Res., **51**, 5851 (1991). Besides its lower potential for inducing carcinogenesis via formation of DNA adducts which can damage DNA, other advantages of IDX compared with TMX are that IDX has reduced residual estrogenic activity in rats and an improved metabolic profile. Thus, another preferred embodiment of the invention includes the use of a compound of formula (I) which includes when R<sup>4</sup> together with R<sup>3</sup> is -CH<sub>2</sub>-CH<sub>2</sub>- or -S-, or R<sup>5</sup>

is OH, that has reduced, or no, estrogenic activity. The estrogenic activity of an agent or a compound of formula (I) can be determined by methods well known to the art. A more preferred embodiment of the invention includes the use of a compound of formula (I) which includes when R<sup>4</sup> together with R<sup>3</sup> is -CH<sub>2</sub>-CH<sub>2</sub>- or -S-, or R<sup>5</sup> is OH, that forms DNA adducts at low frequency, or preferably not at all, and has low, or preferably no, estrogenic activity. IDX is a preferred embodiment of the present invention.

Also included within the scope of the term tamoxifen are the TMX structural analogs toremifene and raloxifene, metabolites or pharmaceutically acceptable salts thereof. Other "antisteroids" or "steroidal antagonists" can also be useful as agents that increase the level of TGF-beta or lead compounds, including other known stilbene-type antisteroids including cis- and trans-clomiphene, droloxifene, (1-[4-(2-dimethylaminoethoxy)phenyl]-1-(3-hydroxyphenyl)-2-phenyl-2-butene (see U.S. Patent No. 5,384,332), 1-nitro-1-phenyl-2-(4-hydroxyphenyl or anisyl)-2-[4-(2-pyrrol-N-ylethoxy)-phenyl]ethylene (CN-55,945), trans-1,2-dimethyl-1,2-(4-hydroxyphenyl)ethylene (trans-dimethylstilboestrol), trans-diethylstilboestrol, and 1-nitro-1-phenyl-2-(4-hydroxyphenyl)-2-[4-(3-dimethylaminopropoxy)phenyl]-ethylene (GI680).

Known 1,2-diphenylethane-type antisteroids include cis-1,2-anisyl-1-[4-(2-diethylaminoethoxy)phenyl]ethane (MRL-37), 1-(4-chlorophenyl)-1-[4-(2-diethylaminoethoxy)phenyl]-2-phenylethanol (WSM-4613); 1-phenyl-1-[4-(2-diethylaminoethoxy)phenyl]-2-anisylethanol (MER-25); 1-phenyl-1-[4-(2-diethylaminoethoxy)phenyl]-2-anisyl-ethane, mesobutoestrol (trans-1,2-dimethyl-1,2-(4-hydroxyphenyl)-ethane), meso-hexestrol, (+)hexestrol and (-)-hexestrol.

Known naphthalene-type antisteroids include nafoxidine, 1-[4-(2,3-dihydroxypropoxy)phenyl]-2-phenyl-6-hydroxy-1,2,3,4-tetrahydro-naphthalene, 1-(4-hydroxyphenyl)-2-phenyl-6-hydroxy-1,2,3,4-tetrahydronaphthalene, 1-[4-(2-pyrrol-N-ylethoxy)-phenyl]-2-phenyl-6-methoxy-3,4-dihydronaphthalene (U11, 100A), and 1-

[4-(2,3-dihydroxypropoxy)phenyl]-2-phenyl-6-methoxy-3,4-dihydronaphthalene (U-23, 469).

Known antisteroids which do not fall anywhere within these structural classifications include coumetstrol, biochanin-A, genistein, methallenstil, phenocytin, and 1-[4-(2-dimethylaminoethoxy)phenyl]-2-phenyl-5-methoxyindene (U, 11555). In the nomenclature employed hereinabove, the term "anisyl" is intended to refer to a 4-methoxyphenyl group.

The pharmaceutically acceptable inorganic and organic acid amine salts of the amino group-containing antisteroids are also included within the scope of the term "antisteroid", as used herein, and include citrates, tartrates, acetates, hydrochlorides, hydrosulfates and the like.

"TGF-beta" includes transforming growth factor-beta as well as functional equivalents, derivatives and analogs thereof. The TGF-beta isoforms are a family of multifunctional, disulfide-linked dimeric polypeptides that affect activity, proliferation and differentiation of various cells types. TGF-beta is a polypeptide produced in a latent propeptide form having, at this time, no identified biological activity. To be rendered active and, therefore, capable of inhibiting vascular smooth muscle cell proliferation, the propeptide form of TGF-beta must be cleaved to yield active TGF-beta.

"TGF-beta activator" includes moieties capable of directly or indirectly activating the latent form of TGF-beta to the active form thereof. A number of the compounds of formula (I) are believed to be TGF-beta activators.

"TGF-beta production stimulator" includes moieties capable of directly or indirectly stimulating the production of TGF-beta (generally the latent form thereof). Such TGF-beta production stimulators may be TGF-beta mRNA regulators (i.e., moieties that increase the production of TGF-beta mRNA), enhancers of TGF-beta mRNA expression or the like.

"Direct" action includes, but is not limited to, an agent which acts to increase active TGF-beta levels. For example, direct action indicates that cells upon which the agent acts increase TGF-beta mRNA production increase the

cleavage of latent TGF-beta, or that the agent increases the level of TGF-beta which is capable of binding to its receptor.

"Indirect" action of an agent includes, but is not limited to, an agent of the invention that acts through one or more other moieties acts to increase the  
5 level of active TGF-beta. For example, an agent that acts through one or more other moieties to release TGF-beta from complexes that inhibit or prevent the binding of active TGF-beta to its receptor, or an agent that acts through one or more other moieties to stimulate the production of TGF-beta mRNA or the expression of TGF-beta, acts indirectly.

10 "Sustained release" means a dosage form designed to release a therapeutic agent therefrom for a time period ranging from about 3 to about 21 days. Release over a longer time period is also contemplated as a "sustained release" dosage form of the present invention.

For the purposes of this description, the prototypical cells, upon which  
15 the effects of an agent that increases the level of TGF-beta are felt, are smooth muscle cells, endothelial cells and pericytes derived from the medial layers of vessels which proliferate in intimal hyperplastic vascular sites following injury, such as that caused during PTCA. An agent that increases the level of TGF-beta is not restricted in use for therapy following angioplasty; rather, the usefulness  
20 thereof will be proscribed by their ability to inhibit abnormal cellular proliferation, for example, of smooth muscle cells, endothelial cells and pericytes in the vascular wall. Thus, other aspects of the invention include agents, which increase the level of TGF-beta, used in early therapeutic intervention for reducing, delaying, or eliminating (and even reversing)  
25 atherosclerotic plaque formation and areas of vascular wall hypertrophy and/or hyperplasia. Agents which increase the level of TGF-beta also find utility for early intervention in pre-atherosclerotic conditions, e.g., they are useful in patients at a high risk of developing atherosclerosis or with signs of hypertension resulting from atherosclerotic changes in vessels or vessel stenosis due to  
30 hypertrophy of the vessel wall.



Agents which increase the level of TGF-beta also find utility in the treatment of diabetics with decreased levels of TGF-beta (as described hereinbelow), particularly in diabetics at risk of, or afflicted with, vascular disease. One example of a vascular disease which afflicts certain diabetics is diabetic retinopathy, where angiogenesis results in blindness over a 3-6 month period.

Diabetic retinopathy is one of the most serious complications of diabetes mellitus, and a major cause of blindness all over the world. In spite of the wide clinical variation of the different stages of diabetic retinopathy, there are generally three processes that are known or thought to be of pathogenetic importance. The first is usually characterized by microangiopathy, ischemia and hypoxia. Visible signs of this process include capillary obliteration or nonperfusion, arteriolar-venular shunt, hyperaggregation of red cells and platelets, sluggish blood flow and an impaired ability of red cells to release oxygen. The second process involves abnormal metabolism of carbohydrate, protein and arachidonic acid. The third process of diabetic retinopathy is thought to involve lipid peroxidation of the retinal membrane, possibly oxygen radical-induced. Although many of these characteristics of diabetic retinopathy are known, effective prevention and therapy for this disease has not been available prior to the present invention.

Agents which increase the level of TGF-beta are useful for inhibiting the pathological proliferation of vascular smooth muscle cells or endothelial cells, e.g., for reducing, delaying, or eliminating stenosis following angioplasty. As used herein the term "reducing" means decreasing the intimal thickening that results from stimulation of smooth muscle cell proliferation following angioplasty, either in an animal model or in man. "Delaying" means delaying the time until onset of visible intimal hyperplasia (e.g., observed histologically or by angiographic examination) following angioplasty and may also be accompanied by "reduced" restenosis. "Eliminating" restenosis following angioplasty means completely "reducing" intimal thickening and/or completely "delaying" intimal hyperplasia in a patient to an extent which makes it no longer

necessary to surgically intervene, i.e., to re-establish a suitable blood flow through the vessel by repeat angioplasty, atheroectomy, or coronary artery bypass surgery. The effects of reducing, delaying, or eliminating stenosis may be determined by methods routine to those skilled in the art including, but not  
5 limited to, angiography, ultrasonic evaluation, fluoroscopic imaging, fiber optic endoscopic examination or biopsy and histology.

The amount of an agent of the invention, i.e., one which increases the level of TGF-beta, administered is selected to treat vascular trauma of differing severity, with smaller doses being sufficient to treat lesser vascular trauma such  
10 as in the prevention of vascular rejection following graft or transplant. Agents which increase the level of TGF-beta that are not characterized by an undesirable systemic toxicity profile at a prophylactic dose are also amenable to chronic use for prophylactic purposes with respect to disease states involving proliferation of vascular smooth muscle cells or endothelial cells over time (e.g., atherosclerosis,  
15 coronary heart disease, thrombosis, myocardial infarction, stroke), preferably via systemic administration. The agents of the invention are not envisioned for the treatment of smooth muscle neoplasms such as leiomyoma and leiomyosarcoma of the bowel and uterus, uterine fibroid or fibroma and the like.

For prevention of restenosis, a series of spaced doses, optionally, in  
20 sustained release dosage form, is preferably administered before and after the traumatic procedure (e.g., angioplasty). The dose may also be delivered locally, via catheter delivered to the afflicted vessel during the procedure. After the traumatic procedure is conducted, a series of follow-up doses are administered over time, preferably in a sustained release dosage form, systemically to  
25 maintain an anti-proliferative effect for a time sufficient to substantially reduce the risk of or to prevent restenosis. A preferred therapeutic protocol duration after angioplasty for this purpose is from about 3 to about 26 weeks.

High levels of lipoprotein Lp(a) are known to constitute a substantial risk factor for atherosclerosis, coronary heart disease and stroke. One symptom  
30 associated with such conditions and other problems, such as restenosis following balloon angioplasty and other pathogenic conditions, is the proliferation or the

migration of smooth muscle cells. No direct link between Lp(a) and proliferation of vascular smooth muscle cells had been established in the prior art.

An *in vivo* pathway for the modulation of vascular smooth muscle cell proliferation is shown in Figure 1. TGF-beta is believed to contribute to the inhibitory mechanism that maintains vascular smooth muscle cells in a non-proliferative state in healthy vessels.

Vascular smooth muscle cell proliferation is inhibited by an active form of TGF-beta. Tamoxifen has been shown by the experimentation detailed in Example 1:hereof to stimulate both the production and the activation of TGF-beta. Heparin stimulates the activation of TGF-beta by affecting the release of the active form of TGF-beta from inactive complexes present in serum. TGF-beta neutralizing antibodies inhibit the activity of TGF-beta, thereby facilitating the proliferation of vascular smooth muscle cells. An apparent *in vivo* physiological regulator of the activation of TGF-beta is plasmin. Plasmin is derived from plasminogen through activation by, for example, TPA (tissue plasminogen activator). Plasmin activity is inhibited by the lipoprotein Lp(a) or apolipoprotein(a) (apo(a)), thereby decreasing the activation of the latent form of TGF-beta and facilitating proliferation of vascular smooth muscle cells.

An additional pathway for the modulation of vascular smooth muscle cell proliferation is shown in Figure 2. Resting smooth muscle cells constitute cells in their normal, quiescent non-proliferative state. Such resting smooth muscle cells may be converted to proliferating smooth muscle cells through activation by platelet derived growth factor (PDGF), fibroblast growth factor (FGF) or other stimulatory moieties. The proliferating smooth muscle cells may be converted to continual proliferating smooth muscle cells (i.e., smooth muscle cells capable of generating a pathological state resulting from over-proliferation thereof) by an autocrine growth factor. This growth factor is believed to be produced by proliferating smooth muscle cells. An increased level of autocrine growth factor, which can be inhibited by the active form of TGF-beta or an

appropriately structured (i.e., designed) small molecule inhibitor, is believed to mediate the production of continual proliferating smooth muscle cells.

Lp(a) consists of low density lipoprotein (LDL) and apo(a). Apo(a) shares approximately 80% amino acid identity with plasminogen (see MacLean et al., Nature, 330: 132, 1987). Lp(a) has been found to inhibit cell-associated plasmin activity (see, for example, Harpel et al., Proc. Natl. Acad. Sci. USA, 86: 3847, 1989). Experiments conducted on human aortic vascular smooth muscle cells derived from healthy transplant donor tissue, cultured in Dulbecco's modified Eagles medium (DMEM) + 10% fetal calf serum (FCS) as described in Kirschenlohr et al., Am. J. Physiol., 265, C571 (1993), indicated the following:

- 1) Addition of Lp(a) to sub-confluent human vascular smooth muscle cells stimulated their proliferation in a dose dependent manner (addition of 500 nM Lp(a) to human vascular smooth muscle cells caused a reduction in doubling time from 82 +/- 4 hours to 47 +/- 4 hours);
- 2) Addition of apo(a) had a similar effect, although a higher concentration of apo(a) appeared to be required therefor;
- 3) Addition of LDL at varying concentrations up to 1 micromolar had no effect on proliferation.

One possible mode of action for Lp(a) and apo(a) is competitive inhibition of surface-associated plasminogen activation, which in turn inhibits the subsequent activation of TGF-beta by plasmin. TGF-beta is a potent growth inhibitor of a number of anchorage-dependent cells, including smooth muscle cells. TGF-beta is produced as a latent propeptide having a covalently linked homodimer structure in which the active moiety is non-covalently linked to the amino-terminal portion of the propeptide. Latent TGF-beta must be cleaved (e.g., *in vitro* by acid treatment or *in vivo* by the serine protease plasmin) in order to become capable of inhibiting the proliferation of vascular smooth muscle cells. Plasmin is therefore a leading candidate to be a physiological regulator of TGF-beta.

The hypothesis that Lp(a) and apo(a) were acting on cultured human vascular smooth muscle cells by interfering with activation of latent TGF-beta

was tested. In support of this hypothesis, an observation was made that plasmin activity associated with vascular smooth muscle cells was reduced 7-fold by Lp(a) and 5-fold by apo(a). The plasmin activity in the conditioned medium was also reduced by Lp(a) and apo(a) by about 2-fold, but was much lower than cell-associated plasmin activity in vascular smooth muscle cell cultures. These observations are consistent with previous findings that Lp(a) is a more potent inhibitor of surface-associated, rather than fluid phase, plasminogen activation.

To exclude the possibility that Lp(a) was affecting the synthesis of plasminogen activators rather than plasminogen activation, plasminogen activator levels in human vascular smooth muscle cell cultures were measured in the presence and absence of the lipoproteins and in the presence of a large excess of plasminogen, so that the lipoproteins present would not significantly act as competitive inhibitors. Total plasminogen activator activity was not affected by the presence of any of the lipoproteins in the vascular smooth muscle cell cultures. For example, plasminogen activator activity in the conditioned medium remained at  $0.7 \pm 0.6$  mU/ml with Lp(a) additions up to 500 nM.

Lp(a) and apo(a) both reduced the level of active TGF-beta by more than 100-fold compared to control or LDL-treated cultures. The level of total latent plus active TGF-beta measured by ELISA as described in Example 8 was unaffected by the presence of Lp(a) or apo(a), however. These facts lead to the conclusion that Lp(a) stimulates proliferation of human vascular smooth muscle cells by inhibiting plasmin activation of latent TGF-beta to active TGF-beta.

To further test this conclusion and exclude the possibility that Lp(a) was acting by binding active TGF-beta as well as reducing plasmin activity, human vascular smooth muscle cells were cultured in the presence of Lp(a). These cells had a population doubling time of  $47 \pm 3$  hours. Addition of plasmin was able to overcome the population doubling time reducing effect of Lp(a) and reduce the cell number to control levels, with the population doubling time increased to  $97 \pm 4$  hours.

The role of plasmin in the pathway was confirmed by studies in which inhibitors of plasmin activity were added to human vascular smooth muscle

cells. Like Lp(a), these protease inhibitors increased cell number. Aprotinin, for example, decreased the population doubling time from 82 +/- 4 hours in control cultures to 48 +/- 5 hours, and alpha2-antiplasmin decreased the population doubling time to 45 +/- 2 hours. 500 nM Lp(a) and aprotinin addition resulted in only a slight additional stimulation of proliferation, with the population doubling time for cultures of this experiment being 45 +/- 6 hours. Neutralizing antibodies to TGF-beta similarly decreased population doubling time in vascular smooth muscle cells (see, for example, Example 1). In summary, Lp(a), plasmin inhibitors and neutralizing antibody to TGF-beta stimulate proliferation of vascular smooth muscle cells, while plasmin nullifies the growth stimulation of Lp(a). These results support the theory that the mode of action of Lp(a) and apo(a) is the competitive inhibition of plasminogen activation.

Experimentation conducted to ascertain the impact of tamoxifen on TGF-beta and vascular smooth muscle cell proliferation is set forth in detail in Example 1. The results of those experiments are summarized below.

- 1) Addition of tamoxifen decreased the rate of proliferation, with maximal inhibition observed at concentrations above 33 micromolar. 50 micromolar tamoxifen concentrations produced a cell number 96 hours following the addition of serum that was reduced by 66% +/- 5.2% (n=3) as compared to cells similarly treated in the absence of tamoxifen.
- 2) Tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing. Inhibition of vascular smooth muscle cells caused by tamoxifen therefore appears to be the result of an increase in the cell cycle time of nearly all (>90%) of the proliferating cells.
- 3) Tamoxifen decreases the rate of proliferation of serum-stimulated vascular smooth muscle cells by increasing the time taken to traverse the G<sub>2</sub> to M phase of the cell cycle.
- 4) Tamoxifen decreased the rate of proliferation of vascular smooth muscle cells by inducing TGF-beta activity.
- 5) Vascular smooth muscle cells produced TGF-beta in response to tamoxifen. Tamoxifen appears to increase TGF-beta activity in cultures of rat

vascular smooth muscle cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

6) Tamoxifen, unlike heparin, does not act by releasing TGF-beta from inactive complexes present in serum.

5        7) TGF-beta mRNA was increased by approximately 10-fold by 24 hours after addition of tamoxifen (10 micromolar). This result suggests that the expression of TGF-beta mRNA by the smooth muscle cells will be increased, thereby facilitating decreased proliferation thereof by activated TGF-beta.

8) Tamoxifen is a selective inhibitor of vascular smooth muscle  
10 proliferation with an  $ED_{50}$  (a concentration resulting in 50% inhibition) at least 10-fold lower for vascular smooth muscle cells than for adventitial fibroblasts.

Additional experimentation has shown that the addition of Lp(a) or apo(a) substantially reduced the rat vascular smooth muscle cell proliferation inhibitory activity of tamoxifen, with the population doubling time in the  
15 presence of tamoxifen and Lp(a) being  $42 \pm 2$  hours (as compared to a population doubling time of  $55 \pm 2$  hours for tamoxifen alone, and a time of  $35 \pm 2$  hours for the control). Also, the presence of Lp(a) reduced the levels of active TGF-beta produced in response to the addition of tamoxifen by about 50-fold. Addition of plasmin to rat vascular smooth muscle cells treated with  
20 tamoxifen and Lp(a) resulted in most of the TGF-beta being activated, and proliferation was again slowed (with the population doubling time being  $57 \pm 3$  hours). These observations are consistent with the theory that Lp(a) acts by inhibiting TGF-beta activation.

Identification of therapeutic agents (direct or indirect TGF-beta activators  
25 or production stimulators) that act to inhibit vascular smooth muscle cell proliferation by the pathway shown in Figure 1 can be identified by a practitioner in the art by conducting experiments of the type described above and in Example 1. Such experimental protocols facilitate the identification of therapeutic agents useful in the practice of the present invention and capable of  
30 one of the following activities:

1) production or activation of TGF-beta:

- 2) having TGF-beta-like activity;
  - 3) activation of plasminogen;
  - 4) increase in plasmin activity; or
  - 5) reduction of Lp(a) or apo(a) level or levels of  $\pi$ -I or other inhibitors of
- 5 TGF-beta activation.

Identification of therapeutic agents (direct or indirect TGF-beta activators or production stimulators) that act to inhibit vascular smooth muscle cell proliferation by the pathway shown in Figure 2 can be identified by a practitioner in the art by conducting experimentation using known techniques

10 that are designed to identify growth factors made by proliferating smooth muscle cells, which growth factors also act on those cells (i.e., autocrine growth factors). Rational drug design can then be used to screen small molecules for the ability to inhibit the production or activity of such autocrine growth factors as lead compounds for drug design. Such experimental protocols facilitate the

15 identification of therapeutic agents useful in the practice of the present invention and capable of one of the following activities:

- 1) production or activation of TGF-beta;
  - 2) having TGF-beta-like activity; or
  - 3) inhibit the activity or production of an autocrine growth factor
- 20 produced by proliferating smooth muscle cells.

Smooth muscle cell proliferation is a pathological factor in myocardial infarctions, atherosclerosis, thrombosis, restenosis and the like. Therapeutic/prophylactic agents of the present invention, including tamoxifen and the like, having at least one of the activities recited above and therefore

25 being capable of inhibiting proliferation of vascular smooth muscle cells, are useful in the prevention or treatment of these conditions. Manipulation of the proliferation modulation pathway for vascular smooth muscle cells to prevent or reduce such proliferation removes or reduces a major component of the arterial lesions of atherosclerosis and the restenosed arteries following angioplasty, for

30 example.



More specifically, chronically maintaining an elevated level of activated TGF-beta reduces the probability of atherosclerotic lesions forming as a result of vascular smooth muscle cell proliferation. Consequently, administration of TGF-beta activators or TGF-beta production stimulators protects against

5 atherosclerosis and subsequent myocardial infarctions that are consequent to coronary artery blockage. Also, substantially increasing the activated TGF-beta level for a short time period allows a recipient to at least partially offset the strong stimulus for vascular smooth muscle cell proliferation caused by highly traumatic injuries or procedures such as angioplasty. Continued delivery to the

10 traumatized site further protects against restenosis resulting from vascular smooth muscle cell proliferation in the traumatized area.

Prevention or treatment relating to a traumatized or diseased vascular site, for example, the TGF-beta activators or production stimulators may also be administered in accordance with the present invention using an infusion catheter,

15 such as produced by C.R. Bard Inc., Billerica, MA, or that disclosed by Wolinsky ( U.S. Patent No. 4,824,436) or Spears (U.S. Patent No. 4,512,762). In this case, a therapeutically/prophylactically effective dosage of the TGF-beta activator or production stimulator will be typically reached when the concentration thereof in the fluid space between the balloons of the catheter is in

20 the range of about  $10^{-3}$  to  $10^{-12}$  M. It is recognized by the present inventors that TGF-beta activators or stimulators may only need to be delivered in an anti-proliferative therapeutic/prophylactic dosage sufficient to expose the proximal (6 to 9) cell layers of the intimal or tunica media cells lining the lumen thereto. Also, such a dosage can be determined empirically, e.g., by a) infusing vessels

25 from suitable animal model systems and using immunohistochemical methods to detect the TGF-beta activator or production stimulator and its effects; and b) conducting suitable *in vitro* studies.

It will be recognized by those skilled in the art that desired therapeutically/prophylactically effective dosages of a TGF-beta activator or

30 production stimulator administered by a catheter in accordance with the invention will be dependent on several factors, including, e.g.: a) the

atmospheric pressure applied during infusion; b) the time over which the TGF-beta-activator or production stimulator administered resides at the vascular site; c) the nature of the therapeutic or prophylactic agent employed; and/or d) the nature of the vascular trauma and therapy desired. Those skilled practitioners  
5 trained to deliver drugs at therapeutically or prophylactically effective dosages (e.g., by monitoring drug levels and observing clinical effects in patients) will determine the optimal dosage for an individual patient based on experience and professional judgment. In a preferred embodiment, about 0.3 atm (i.e., 300 mm of Hg) to about 5 atm of pressure applied for 15 seconds to 3 minutes directly to  
10 the vascular wall is adequate to achieve infiltration of a TGF-beta activator or production stimulator into the smooth muscle layers of a mammalian artery wall. Those skilled in the art will recognize that infiltration of the TGF-beta activator or production stimulator into intimal layers of a diseased human vessel wall in free or sustained-release form will probably be variable and will need to be  
15 determined on an individual basis.

While two representative embodiments of the invention relate to prophylactic or therapeutic methods employing an oral dosage form or infusion catheter administration, it will be recognized that other methods for drug delivery or routes of administration may also be useful, e.g., injection by the  
20 intravenous, intralymphatic, intrathecal, intraarterial, local delivery by implanted osmotic pumps or other intracavity routes. Administration of TGF-beta activators or production stimulators in accordance with the present invention may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic  
25 or prophylactic and other factors known to skilled practitioners.

In the practice of certain embodiments of the present invention, catheter administration routes including systemic and localized delivery to the target site are preferably conducted using a TGF-beta activator or production stimulator dispersed in a pharmaceutically acceptable carrier. Tamoxifen and its structural  
30 analogs and salts, including the compounds of formula (I) can be administered by a variety of routes including oral, rectal, transdermal, subcutaneous.

intravenous, intramuscular, and intranasal. These compounds preferably are formulated prior to administration, the selection of which will be decided by the attending physician. Typically, TMX and its structural analogs and salts, including the compounds of formula (I), or a pharmaceutically acceptable salt thereof, is combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage form.

The total active ingredients in such formulations comprises from 0.1 to 99.9% by weight of the formulation. By "pharmaceutically acceptable" it is meant the carrier, diluent, excipient, and/or salt must be compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof.

Pharmaceutical formulations containing TMX and its structural analogs and salts, including the compounds of formula (I), can be prepared by procedures known in the art using well known and readily available ingredients. For example, the compounds of formula (I) can be formulated with common excipients, diluents, or carriers, and formed into tablets, capsules, suspensions, powders, and the like. Examples of excipients, diluents, and carriers that are suitable for such formulations include the following fillers and extenders such as starch, sugars, mannitol, and silicic derivatives; binding agents such as carboxymethyl cellulose and other cellulose derivatives, alginates, gelatin, and polyvinyl-pyrrolidone; moisturizing agents such as glycerol; disintegrating agents such as calcium carbonate and sodium bicarbonate; agents for retarding dissolution such as paraffin; resorption accelerators such as quaternary ammonium compounds; surface active agents such as cetyl alcohol, glycerol monostearate; adsorptive carriers such as kaolin and bentonite; and lubricants such as talc, calcium and magnesium stearate, and solid polyethyl glycols.

The compounds also can be formulated as elixirs or solutions for convenient oral administration or as solutions appropriate for parenteral administration, for example, by intramuscular, subcutaneous or intravenous routes.

The present invention also contemplates therapeutic methods and therapeutic dosage forms involving sustained release of the TGF-beta activator

or production stimulator to target cells. Preferably, the target cells are vascular smooth muscle cells, cancer cells, somatic cells requiring modulation to ameliorate a disease state and cells involved in immune system-mediated diseases that are accessible by local administration of the dosage form.

- 5 Consequently, the methods and dosage forms of this aspect of the present invention are useful for inhibiting vascular smooth muscle cells in a mammalian host, employing a therapeutic agent that inhibits the activity of the cell (e.g., proliferation, formation of lipid proliferative lesions, contraction, migration or the like) but does not kill the cell and, optionally, a vascular smooth muscle cell
- 10 binding protein. Sustained released dosage forms for systemic administration as well as for local administration are also employed in the practice of the present method. Formulations intended for the controlled release of pharmaceutically-active compounds *in vivo* include solid particles of the active ingredient that are coated or tableted with film-forming polymers, waxes, fats, silica, and the like.
- 15 These substances are intended to inhibit the dissolution, dispersion or absorption of the active ingredient *in vivo*. Hydroxypropylmethyl cellulose is one example of an ingredient that can provide a slow or controlled release of the active ingredient. The compounds can also be delivered via patches for transdermal delivery, subcutaneous implants, infusion pumps or via release from implanted
- 20 sustained release dosage forms.

- Another embodiment of the invention relates to prophylactic or therapeutic "sustained release" methods from the surface of an intravascular device employing an excipient matrix which will release the TGF-beta activators over a one-week to two-year or longer period. The surface coating and the
- 25 impregnated forms of the article can be a biodegradable or nonbiodegradable polymer or ceramic material which will slowly release the TGF-beta activator at a dose rate that will inhibit the proliferation of fibromuscular cells and/or lipid accumulation which would impair the function of the device. The accumulation of fibromuscular cells, including VSMC, and their associated matrix, along with
- 30 lipid containing foam cells can decrease the luminal area of intravascular stents, synthetic grafts and indwelling catheters to an extent that blood flow is critically

impaired and the device can fail functionally. The inhibition of this proliferation would extend the clinically functional life of these devices and be of significant clinical benefit to the patients.

The sustained release dosage forms of this embodiment of the invention  
5 needs to deliver a sufficient anti-proliferative, preferably cytostatic, dosage to expose cells immediately adjacent to the device surface to be therapeutic. This would inhibit cellular attachment, migration and proliferation of the fibromuscular cells and foam cells. This dosage is determinable empirically by implanting a specific device intravascularly with variable amounts of the TGF-  
10 beta activator and modification of the polymer excipient, both of which would affect the rate and duration of the drug release required to achieve the cytostatic dosing which has been demonstrated in vascular smooth muscle cell tissue culture experiments. Different types of devices may require different periods of therapeutic drug release. For example, the use in grafts and stents are considered  
15 permanently implanted devices; however, it may not be necessary to have the active agent continuously released from the device. It appears from initial observations that if excessive proliferation is prevented until the graft or stent is surrounded by quiescent tissue and covered by intact endothelium then continued release of cytostatic agents may be unnecessary. Devices such as indwelling  
20 catheters, however, do not become embedded in quiescent vascular wall tissue and overgrown with endothelium. These devices may require the continual release of drugs to suppress the proliferation of tissue over their external and luminal surfaces. To achieve this prolonged period of sustained drug release, larger amounts of agent and different types of, or modification of, the polymer or  
25 excipient are preferable.

The sustained release dosage forms of the present invention, particularly, for local administration, are preferably either non-degradable microparticulates or nanoparticulates or biodegradable microparticulates or nanoparticulates. More preferably, the microparticles or nanoparticles are formed of a polymer  
30 containing matrix that biodegrades by random, nonenzymatic, hydrolytic scissioning. A particularly preferred structure is formed of a mixture of

thermoplastic polyesters (e.g., polylactide or polyglycolide) or a copolymer of lactide and glycolide components. The lactide/glycolide structure has the added advantage that biodegradation thereof forms lactic acid and glycolic acid, both normal metabolic products of mammals.

5           Therapeutic dosage forms (sustained release-type) of the present invention exhibit the capability to deliver therapeutic agent to target cells over a sustained period of time. Such dosage forms are disclosed in co-pending U.S. patent application Serial No. 08/241,844, filed May 12, 1994, which is a continuation-in-part of Serial No. 08/62,451, filed May 13, 1993, which is in  
10          turn a continuation-in-part of Serial No. 08/011,669, which is in turn a continuation-in-part of PCT application US 92/08220, filed September 25, 1992. These applications are incorporated by reference herein. Therapeutic dosage forms of this aspect of the present invention may be of any configuration suitable for this purpose. Preferred sustained release therapeutic dosage forms exhibit  
15          one or more of the following characteristics:

- microparticulate (e.g., from about 0.5 micrometers to about 100 micrometers in diameter, with from about 0.5 to about 2 micrometers more preferred) or nanoparticulate (e.g., from about 1.0 nanometer to about 1000 nanometers in diameter, with from about 50 to about 250 nanometers more  
20          preferred), free flowing powder structure;
- biodegradable structure designed to biodegrade over a period of time between from about 3 to about 180 days, with from about 10 to about 21 days more preferred, or nonbiodegradable structure to allow therapeutic agent diffusion to occur over a time period of between from about 3 to about 180 days,  
25          with from about 10 to about 21 days preferred;
- biocompatible with target tissue and the local physiological environment into which the dosage form is being administered, including biocompatible biodegradation products;
- facilitate a stable and reproducible dispersion of therapeutic agent  
30          therein, preferably to form a therapeutic agent-polymer matrix, with active therapeutic agent release occurring through one or both of the following routes:

(1) diffusion of the therapeutic agent through the dosage form (when the therapeutic agent is soluble in the polymer or polymer mixture forming the dosage form); or (2) release of the therapeutic agent as the dosage form biodegrades; and

- 5       - capability to bind with one or more cellular and/or interstitial matrix epitopes, with from about 1 to about 10,000 binding protein/peptide-dosage form bonds preferred and with a maximum of about 1 binding peptide-dosage form per 150 square angstroms of particle surface area more preferred. The total number bound depends upon the particle size used. The binding proteins or
- 10 peptides are capable of coupling to the particulate therapeutic dosage form through covalent ligand sandwich or non-covalent modalities as set forth herein.

For example, nanoparticles containing a compound of the formula (I) may be prepared using biodegradable polymers including poly(D,L-lactic acid)PLA, poly(D,L-lactic-co-glycolic) PLGA, methacrylic acid copolymer,

15 poly(epsilon-caprolactone), using either 1) n-solvent emulsification-evaporation techniques or 2) emulsification - precipitation techniques. These processes involve dispersion of polymer in an organic solvent (e.g., acetone or benzyl alcohol) with or without a co-solvent, typically methylene chloride. The compound of formula (I) is contained in the organic solvent. In some cases,

20 solvents are then mixed and then added dropwise to an aqueous solution containing stabilizing hydrocolloid [e.g., poly(vinyl alcohol) or gelatin] (i.e., oil in water) with mechanical agitation or sonication. Following formation of the stable emulsion, the chlorinated solvent is removed via evaporation of the stirred emulsion, yielding nanoparticles that then can be freed of organic solvents by

25 tangential filtration or repeated washings by centrifugation/resuspension. The resultant aqueous suspension can then be frozen with or without saccharide or other cryoprotectants and lyophilized to yield nanoparticles capable of resuspension in physiological salt solutions with simple agitation or sonication.

Alternatively, the aqueous solution can be added with agitation or

30 sonication to the organic phase lacking chlorinated solvent (i.e., water-in-oil emulsion) followed by further addition of aqueous solution to achieve a phase

inversion, to precipitate the nanoparticles. Alternatively, precipitation can be augmented by addition to salting-out agents in the aqueous solvent. Typically, for emulsification-evaporation technique 750 mg PLGA can be dissolved in 30 mL of methylene chloride. Five mL of methylene chloride containing 75 mg of a compound of formula (I), for example, tamoxifen, is added. This organic phase is added dropwise to 180 mL of aqueous solution of 2.5% poly(vinyl alcohol, PVP) (20-70 kD mol Wt.) with sonication using a Branson 450 sonifier at 15-55 watt output, for approximately 10 minutes to form a soluble emulsion. Sonication is performed in an ice bath at a temperature not exceeding 15°C. the emulsion is then further stirred at room temperature for 24 hours to allow for evaporation of the chlorinated solvent. The resultant nanoparticles are purified further using a Sartorius targeted filtration device fitted with a 100 mm pore polyolefin cartridge filter. For the emulsification-precipitation technique, 10 mL of aqueous PMP (10-30% w/w) is added, under mechanical stirring at 1200-5000 rpm, to 5 mL of benzyl alcohol containing 10-15% w/w polymer PLA or PLGA and 10-15 w/w of a compound of the formula (I), for example, tamoxifen, following oil-in-water emulsion formation over 5 minutes. Water (160 mL) is then added to effect a phase inversion, resulting in diffusion of organic solvent into the water with concomitant precipitation of polymer as solid nanoparticles in the ensuing 10 minutes.

For TGF-beta activators or production stimulators, such as compounds of the formula (I), several exemplary dosing regimens are contemplated, depending upon the condition being treated and the stage to which the condition has progressed. For prophylactic purposes with respect to atherosclerosis, for example, a low chronic dose sufficient to elevate *in vivo* TGF-beta production is contemplated. An exemplary dose of this type is about 0.1 mg/kg/day (ranging between about 0.1 and about 10 mg/kg/day), preferably about 0.1-1.0 mg/kg/day, most preferably about 0.3 mg/kg/day. Another exemplary dose range is from about 0.01 to about 1000 micrograms/ml. Such low doses are also contemplated for use with respect to ameliorating stenosis following relatively low trauma



injury or intervention, such as vein grafts or transplants or organ allografts, for example.

For prevention of restenosis following angioplasty, an alternative dosing regimen is contemplated which involves a single "pre-loading" dose (or multiple, 5 smaller pre-loading doses) given before or at the time of the intervention, with a chronic smaller (follow up) dose delivered daily for two to three weeks or longer following intervention. For example, a single pre-loading dose may be administered about 24 hours prior to intervention, while multiple preloading doses may be administered daily for several days prior to intervention.

10 Alternatively, one or more pre-loading doses may be administered about 1-4 weeks prior to intervention. These doses will be selected so as to maximize TGF-beta activator or production stimulator activity, while minimizing induction of synthesis and secretion of extracellular matrix proteins. Such a dosing regimen may involve a systemic pre-loading dose followed by a sustained 15 release chronic dose, or the sustained release dosage form may be designed to deliver a large dose over a short time interval as well as a smaller chronic dose for the desired time period thereafter. Some nausea may be encountered at the higher dose; however, the use of a sustained release or other targeted dosage form is expected to obviate this side effect, because the recipient will not be 20 subjected to a high systemic dose of the therapeutic agent.

The local particulate dosage form administration may also localize to normal tissues that have been stimulated to proliferate, thereby reducing or eliminating such pathological (i.e., hyperactive) conditions. An example of this embodiment of the present invention involves intraocular administration of a 25 particulate dosage form coated with a binding protein or peptide that localizes to pericytes and smooth muscle cells of neovascularizing tissue. Proliferation of these pericytes causes degenerative eye disease. Preferred dosage forms of the present invention release compounds capable of suppressing the pathological proliferation of the target cell population. The preferred dosage forms can also 30 release compounds that increase vessel lumen area and blood flow, reducing the pathological alterations produced by this reduced blood supply.

It will be recognized that where the TGF-beta activator or production stimulator is to be delivered with an infusion catheter, the therapeutic dosage required to achieve the desired inhibitory activity can be anticipated through the use of *in vitro* studies. In a preferred aspect, the infusion catheter may be

5 conveniently a double balloon or quadruple balloon catheter with a permeable membrane. In one representative embodiment, a therapeutically effective dosage of a TGF-beta activator or production stimulator is useful in treating vascular trauma resulting from disease (e.g., atherosclerosis, aneurysm, or the like) or vascular surgical procedures such as angioplasty, atheroectomy, placement of a

10 stent (e.g., in a vessel), thrombectomy, and grafting. Atheroectomy may be performed, for example, by surgical excision, ultrasound or laser treatment, or by high pressure fluid flow. Grafting may be, for example, vascular grafting using natural or synthetic materials or surgical anastomosis of vessels such as, e.g., during organ grafting. Those skilled in the art will recognize that the appropriate

15 therapeutic dosage for a given vascular surgical procedure (above) is determined in *in vitro* and *in vivo* animal model studies, and in human preclinical trials.

Sustained release dosage forms of an embodiment of the invention may only need to be delivered in an anti-proliferative therapeutic dosage sufficient to expose the proximal (6 to 9) cell layers of the tunica media smooth muscle cells

20 lining the lumen to the dosage form. This dosage is determinable empirically, e.g., by a) infusing vessels from suitable animal model systems and using immunohistochemical, fluorescent or electron microscopy methods to detect the dosage form and its effects; and b) conducting suitable *in vitro* studies.

In a representative example, this therapeutically effective dosage is

25 achieved by determining in smooth muscle cell tissue culture the pericellular agent dosage, which at a continuous exposure results in a therapeutic effect between the toxic and minimal effective doses. This therapeutic level is obtained *in vivo* by determining the size, number and therapeutic agent concentration and release rate required for particulates infused between the

30 smooth muscle cells of the artery wall to maintain this pericellular therapeutic dosage.

Human vascular smooth muscle cells (VSMC) are more difficult to grow in culture than VSMC derived from other species, such as rat. Medium conditioned on human VSMC decreased the proliferation of rat VSMC *in vitro*. Entry of rat VSMC into S phase of the cell cycle was not affected. However, the duration of G<sub>2</sub> and/or M phase was extended. Anti-TGF-beta antibody reversed the delayed entry into M phase caused by exposure to human VSMC conditioned medium (HCM). An examination of the HCM showed that 64±12% of the TGF-beta present in the medium was already activated. In contrast, rat VSMC conditioned medium displayed very low levels of latent TGF-beta and no detectable TGF-beta activity. Human VSMC were found to produce tissue plasminogen activator (TPA) activity in culture. The TPA leads to an increase in plasmin activity, which in turn activates TGF-beta. This was confirmed by culturing human VSMC in the presence of aprotinin, a plasmin inhibitor. Aprotinin increased the rate of proliferation of human VSMC to almost the same extent as neutralizing anti-TGF-beta antibodies and  $\alpha_2$ -antiplasmin. Thus, growth of human VSMC in culture is determined by the production of TGF-beta activated by plasmin, which feeds back in an autocrine loop to increase the duration of the cell cycle.

Subcultured human aortic VSMC remain more differentiated in culture than rat aorta VSMC (i.e., they contain higher levels of the smooth muscle-specific isoforms of myosin heavy chain (SM-MHC) and  $\alpha$ -actin). TGF-beta likely plays a role in maintaining SM-MHC and  $\alpha$ -actin content, and thus may be responsible for maintaining cells in a more differentiated phenotype. In view of these data, heparin, which is believed to release TGF-beta from inactive complexes in the serum, would be predicted to have little effect on the rate of proliferation of human VSMC, which is already inhibited by endogenous active TGF-beta production. Such observations may explain why human clinical trials of heparin administered after PTCA have failed to demonstrate any beneficial effect.

Freshly dispersed rat aortic VSMC lose SM-MHC and  $\alpha$ -SM actin as they start to proliferate. After 7 days in culture when the cells reach confluence,

serum is removed, and approximately 40% of the VSMC reexpress SM-MHC and  $\alpha$ -SM actin at levels comparable to those present in freshly dispersed cells. If the cells were subcultured for more than five passages and allowed to reach confluence, less than 1% reexpress SM-MHC even after prolonged serum withdrawal. These cells represent proliferating de-differentiated VSMC.

When primary cultures of rat aortic VSMC are exposed to TGF-beta, the loss of the 204 kD (SM-1) and 200 kD (SM-2) SM-MHC isoforms is substantially inhibited. However, TGF-beta did not induce re-expression of SM-MHC in subcultured cells that have very low levels of this protein. Therefore, TGF-beta can maintain a cell's differentiated state (as defined by SM-MHC content), but cannot induce re-differentiation in a de-differentiated proliferating cell. Since TGF-beta extends the G<sub>2</sub> phase of the cell cycle in both primary and passaged VSMC cultures, the data suggest that the pathways that mediate proliferation and differentiation are regulated independently.

Specific markers of both differentiated and proliferating VSMCs have been isolated. Four cell populations were probed using generated cDNAs: (a) freshly dispersed rat aortic cells; (b) freshly dispersed rat aortic VSMC after 7 days in culture (D7 cells); (c) freshly dispersed rat aortic VSMC after subculturing 12 times (S12 cells); and (d) rat fibroblasts. Five classes of gene markers were defined. Class 1 cDNAs were expressed to a similar level in all of the RNAs. Class 2 cDNAs were highly expressed in RNA from freshly dispersed aortic cells, but were barely detectable in D7 or S12 cells and were not detectable in rat fibroblasts. Class 3 cDNAs were expressed at similar levels in freshly dispersed aortic, D7 and S12 cells. Class 4 cDNAs showed higher expression in freshly dispersed aortic and D7 cells than in S12 cells and fibroblasts. Class 5 cDNAs were expressed more strongly in S12 cells than in freshly dispersed aortic cells, D7 cells and fibroblasts. Class 4 genes included  $\alpha$ -SM actin,  $\gamma$ -SM actin, SM22 $\alpha$ , calponin, tropoelastin, phospholamban and CHIP28. In addition, previously defined markers of the differentiated phenotype include SM-MHC, integrin and vinculin. Class 5 genes included matrix G1a (MGP) and osteopontin. When passaged cells were made quiescent by removal

of serum, the levels of MGP and osteopontin did not change significantly, indicating that high expression of these two genes occurs in VSMC that have undergone proliferation, but does not depend on the cells being in the cell cycle.

Such studies of gene expression provide insight into the processes of differentiation that occur during proliferation of VSMC. *In situ* hybridization analysis of balloon-injured rat carotid arteries suggests that dividing intimal cells present 7 days after injury express high levels of both osteopontin and MGP RNA. In contrast, osteopontin is only weakly expressed in the media of intact rat aorta and carotid arteries. Osteopontin and MGP may play a role in regulating calcification, which can occur rapidly in vascular lesions.

In the course of investigating potential heterogeneity of cells from rat aortas, three groups of VSMC clones have been identified. One group consists of small cells that have an epithelioid or cobblestone morphology and proliferate without the need for added growth factors, suggesting production of an autocrine growth factor(s). The second group consists of intermediate size, spindle shaped cells that grow in a characteristic "hills and valleys" pattern and are dependent on exogenous growth factors. These cells resemble the predominant cell morphology in standard cultures of adult aortic VSMC. The third group consists of large, often multinucleate, cells with limited proliferative capacity. These large cells express high quantities of smooth muscle specific proteins.

All three types of cells could be isolated from neonatal and adult rat aortae. However, aortas from young rats yielded high proportions of the small cell clones, while those from adult rats yielded high proportions of intermediate and large cell clones. Clones of small VSMC can be induced to convert to intermediate sized cells by treatment with TGF-beta. A proportion of these cells, in turn, converts to large cells if plated at low density. The small cells may represent a progenitor cell and the large, non-proliferating cells may represent mature VSMC.

VSMC derived from neonatal rat aortas differ from normal adult VSMC in several ways: (a) they do not require exogenous growth factors for sustained growth; (b) they secrete PDGF-like growth factors; (c) they grow with a

characteristic epithelioid morphology; and (d) they express high levels of cytochrome P450IA1, elastin and osteopontin (J. Biol. Chem. 266:3981-86, 1991; Biochem. Biophys. Res. Comm. 177:867-73, 1991; Nature 311:669-71, 1984). After intimal damage, neointimal lesions grow with an epithelioid morphology, secrete a PDGF-like protein and display increased expression of osteopontin in the vascular wall (Proc. Natl. Acad. Sci. USA 83:7311-15, 1986). These data are consistent with the presence *in vivo* of a subpopulation of VSMC that comprises a diminishing proportion of the total cell population with age and which proliferates preferentially.

10 TGF-beta is released by platelets, macrophages and VSMC at sites of vascular injury. Since VSMC and endothelial cells at the site of vascular injury can synthesize and release t-PA, a local mechanism for activating secreted TGF-beta exists. The level of t-PA activity depends on expression of plasminogen activator inhibitor-1 (PAI-1) which is also synthesized in the vessel wall, and  
15 may be up-regulated by TGF-beta. In addition, TGF-beta binds with high affinity to  $\alpha$ 2-macroglobulin. Such binding renders TGF-beta unable to bind to cell surface receptors for TGF-beta. Polyanionic glycosaminoglycans, such as heparin, are also normally present in the vessel wall, and these moieties can reverse the association of TGF-beta with  $\alpha$ 2-macroglobulin. The phenotypic  
20 state of the VSMC may affect the VSMC response to activated TGF-beta. The phenotypic state of the VSMC may be influenced by their extracellular environment. Accordingly, the biological effects of TGF-beta are subject to a variety of regulatory mechanisms.

TGF-beta inhibits DNA synthesis in rat aortic VSMC stimulated with  
25 either PDGF or EGF. In serum stimulated cells, however, TGF-beta has little effect on DNA synthesis. Instead, TGF-beta exerts its anti-proliferative effect by prolonging the G<sub>2</sub> phase of the cell cycle. Likewise, heparin inhibits proliferation of serum-stimulated rat VSMC by extending the G<sub>2</sub> phase of the cell cycle. This effect of heparin can be eliminated by anti-TGF-beta antibody.  
30 These observations suggest that the anti-proliferative effect of heparin on VSMC *in vitro* and possibly *in vivo* may be exerted through the release of TGF-beta.

When VSMC are dispersed in cell culture, they lose contractile proteins and modulate to a "synthetic" phenotype as they proliferate. The majority of VSMC in atheromatous plaques appear to have this synthetic phenotype also. Since loss of smooth muscle-specific proteins occurs spontaneously in cell culture in the absence of mitogens where no proliferation occurs, this phenotypic change is not attributable to mitogenic stimulation, but rather to removal of the cells from their extracellular matrix. The matrix contains large quantities of collagen and glycosaminoglycans that may maintain VSMC in a contractile state. TGF-beta does not exert its anti-proliferative effect through inhibition of phenotypic modulation, however, since it is effective at slowing proliferation of passaged cells that can no longer express contractile proteins. Thus, TGF-beta displays the independent properties of (1) maintaining differentiated adult VSMC in the contractile phenotype; (2) causing maturation of small VSMC to intermediate size, spindle-shaped VSMC; and (3) inhibiting VSMC proliferation regardless of phenotype. Change from a contractile to synthetic phenotype is not obligatory for proliferation.

Cultured VSMC synthesize and secrete large quantities of extracellular matrix proteins. TGF-beta enhances production of extracellular matrix proteins, which favors maintenance of the synthetic phenotype in cells that have been allowed to modulate. In addition, TGF-beta increases expression of numerous protease inhibitors, which also increase accumulation of extracellular matrix proteins.

In hypertension, there is increased thickness of the vessel media, with a consequent decrease in maximum lumen diameter, leading to increased vascular resistance. The increased thickness of the vessel media is due to growth of VSMC within the media. In large conductance vessels, such as the aorta, the VSMC growth is believed to be attributable primarily to VSMC hypertrophy (i.e., enlargement of the cell without proliferation). In hypertensive animals, these vessels display an increased incidence of polyploid cells within the aortic media. In resistance vessels, such as the mesenteric arteries, however, VSMC proliferation may contribute to the increased thickness of the vessel media.

Previously, VSMC growth in hypertension was believed to result from elevated blood pressure. Current data suggest that increased vascular tone and VSMC hypertrophy and/or hyperplasia may be caused independently by a common stimulus. For instance, under certain circumstances, the vasoconstrictor peptide AII may be mitogenic for VSMC. Further, VSMC stimulated with AII also synthesize TGF-beta. Thus, any mitogenic effect of AII might be inhibited by TGF-beta, with the net effect of AII stimulation being arrest in G<sub>1</sub> and hypertrophy without proliferation. AII may induce activation of TGF-beta by stimulating expression of t-PA by VSMC.

10 The VSMC involved in hypertension remain within the media of the vessel and are surrounded by a heparin-containing extracellular matrix. Therefore, any TGF-beta produced is freely available and will maintain VSMC in a contractile state.

In obliterative vascular disease, such as atherosclerosis, VSMC migrate from the media and proliferate in the intima. There they secrete extracellular matrix proteins and form a lipid-rich plaque that encroaches on the vascular lumen. This process is similar to, but slower than, the process that occurs following PTCA, leading to restenosis. Such inappropriate intimal VSMC proliferation also occurs in vascular bypass grafts and the arteries of transplanted organs, leading to graft occlusion and organ failure, respectively. In atherosclerosis, the VSMC involved in the lesion are generally of the synthetic phenotype and localized in the intima, in contrast to the VSMC involved in hypertension.

For medial VSMC involved in atherosclerosis, VSMC migration is accompanied by an increase in synthesis and secretion of matrix proteins and by proliferation. TGF-beta may reduce or prevent the VSMC proliferative response to mitogens and/or may induce synthesis and secretion of extracellular matrix proteins. The effect of TGF-beta in this case would be reduction of cellularity and increase of the matrix component of an atherosclerotic plaque.

30 Alternatively, VSMC in the intima may arise from a population of neonatal-like VSMC that are capable of migration and preferential proliferation



following vascular injury. This intimal phenotype may be either induced or selected in response to vessel injury. When these cells are exposed to TGF-beta, the neonatal-like, small cell phenotype should convert into intermediate sized, spindle-shaped cells that no longer produce an autocrine growth factor. Thus, 5 cells of the intermediate size should have a decreased tendency to proliferate. Over time, a portion of this intermediate sized population of cells would convert to the large, non-proliferative VSMC phenotype.

If VSMC are producing autocrine TGF-beta, tamoxifen has minimal or no further inhibitory effect on VSMC proliferation. Moreover, these TGF-beta- 10 producing VSMC exhibit responses to mitogenic stimuli that may differ from those of VSMC that are not producing TGF-beta. Such data provides further evidence of a complex interaction between the elements that are likely involved in atherosclerosis and vascular injury or trauma.

Transgenic mice that express the human apo(a) gene are useful tools for 15 studying TGF-beta activation, VSMC proliferation and vascular lesions that mimic early human atherosclerotic lesions. In these mice, the apo(a) accumulates in focal regions in the luminal surface of vessel walls. These foci of apo(a) inhibit plasminogen activation, which leads to a decrease in production of plasmin. A low local concentration of plasmin results in reduced activation of 20 TGF-beta. This inhibition of TGF-beta activation is greatest at sites of highest apo(a) accumulation. Further, these effects are observed whether the transgenic mice are fed a normal diet or a lipid-rich diet. Serum levels of activated TGF-beta correlate with the immunofluorescence determinations performed on tissue sections. Osteopontin, a marker of activated VSMC, co-localized with focal 25 apo(a) accumulation and regions of very low TGF-beta activation.

The formation of the atherosclerotic lesion can occur in five stages:

1. MIGRATION. In a healthy vessel, most or all of the smooth muscle cells (SMC) are contained in the vessel media. The appearance of SMC in the enlarged intima during lesion formation must therefore require migration 30 of the SMC from the media to the intima of the vessel. Inhibition of this SMC

migration would significantly alter the nature of the lesion, and may ameliorate the pathology associated with lesion formation.

2. **LIPID ACCUMULATION.** Medial SMC in healthy vessel walls do not significantly accumulate lipid. However, intimal SMC have an increased capacity for lipid uptake and storage. When exposed to elevated levels of circulating lipid (particularly low density lipoprotein; LDL), SMC may become saturated with fatty lipid and die. The accumulation of lipid is necessary for the progression of the lesion to clinical significance, since it forms the thrombogenic necrotic core of the lesion. Inhibition of lipid accumulation in the SMC should significantly reduce or prevent lesion formation and/or progression, thus reducing or preventing atherosclerosis and resultant myocardial infarction.

3. **RECRUITMENT OF INFLAMMATORY CELLS.** Human lesions contain many macrophage-derived cells. The process of recruitment, the function of these cells, and their contribution to pathology are unclear. An oversimplified mechanism suggests that macrophages are attracted to the lipid accumulating in the lesion, in order to remove the lipid from the vessel wall. While inhibition of recruitment of macrophage-derived cells might reduce lesion pathology, it may also speed progression to the lipid-filled, rupture-prone state.

4. **PROLIFERATION.** Intimal SMC accumulation is accompanied by medial thinning in many cases. Therefore, total SMC number may not increase significantly at the lesion site. Furthermore, the chronic nature of atherosclerosis makes it difficult to detect stimulation of proliferation in these lesions. Data obtained from transgenic apo(a) mice suggest that apo(a) may stimulate SMC proliferation. However, evidence that SMC hyperplasia is the major contributor to atherosclerosis is lacking. Thus, the ultimate effect that inhibition of apo(a) has on atherosclerosis is dependent on the contribution of SMC proliferation to initiation or progression of an atherosclerotic plaque.

5. **EXTRACELLULAR MATRIX DEPOSITION.** Atherosclerotic lesions are also rich in extracellular matrix (ECM), and in particular, collagen fibers. Increased ECM synthesis may increase plaque stability. Early plaque rupture, leading to myocardial infarction, may be associated with low ECM

deposition and resultant weakening of the fibrous cap that overlays the necrotic, lipid-rich core of the lesion.

Accordingly, atherosclerosis involves the complex interplay of various processes, some of which may be yet unidentified. Targeting a single process in an effort to reduce or prevent atherosclerosis depends on knowledge of the relative contribution of each process to the manifested pathology. For these reasons, a coordinated, therapeutic strategy is preferred. An exemplary strategy involves inhibition of SMC migration, lipid accumulation and proliferation, with possible beneficial effects of increasing ECM deposition.

10 A diagnostic assay for identifying patients at risk for atherosclerosis, and therefore for identifying suitable candidates for therapy, is also an embodiment of the invention. In addition, this diagnostic assay provides a means to monitor patients that are being treated for atherosclerosis. In one format, a sandwich ELISA for determining total TGF-beta, ELISA plates are coated with an antibody that binds both latent and active TGF-beta. Patient sera are incubated with these ELISA plates, then the plates are washed to remove unbound components of the patients' sera. Rabbit anti-TGF-beta antibody, capable of binding both latent and active TGF-beta, is then added to the plates and incubated. The plates are then washed to remove unbound antibody, and peroxidase-labeled anti-rabbit IgG is added. After incubation and washing, the plates are exposed to the chromogenic substrate, orthophenylenediamine. The presence of total TGF-beta in patients' sera is then determined colorimetrically at  $A_{492}$  by comparison to a standard curve. In patients treated with an agent that modifies TGF-beta, a pretreatment determination of TGF-beta can be compared with post-treatment time points to monitor treatment results and effectiveness.

In an alternate format, TGF-beta type II receptor extracellular domain, which recognizes the active form of TGF-beta, is coated onto ELISA plates. Patient sera are added to the plates, and processed as above. This assay measures active TGF-beta present in sera.

30 In another alternate format, fluorescent-labeled anti-TGF-beta antibody or TGF-beta type II receptor extracellular domain is used in place of peroxidase

labeled second antibody to detect the presence of TGF-beta in patients' sera. In yet another alternate format, anti-TGF-beta antibody or TGF-beta type II receptor extracellular domain is labeled with a radioactive moiety capable of detection by standard means. These latter two assays may be performed in an ELISA format, with or without using the additional anti-TGF-beta antibody described above. In addition, these latter two assays are amenable to other automated or non-automated assay and detection methods.

To determine whether an agent is a TGF-beta activator or TGF-beta production stimulator, an agent or mixture of agents is first tested on rat aortic vascular smooth muscle cells (rVSMCs) for their ability to stimulate the production of active TGF- $\beta$  in the culture medium as originally described for tamoxifen. See Grainger et al. (Biochem. J., 294, 109 (1993)). The key step in demonstrating that cells have a reduced proliferation rate as a result of TGF- $\beta$  production and activation is that the effect can be fully reversed by neutralizing antibodies to TGF- $\beta$ . Incomplete reversal of a decreased rate of proliferation is evidence for TGF- $\beta$  independent effect(s), which may include toxicity. The effects of an agent are then tested on explant human aortic smooth muscle cells (hVSMC) as described in Example 3 to determine whether the agent also stimulates production of TGF- $\beta$  by these cells. The use of explant hVSMCs, prepared and grown as described in Example 3, is essential because (i) explant hVSMCs grown under non-optimal conditions (particularly at low cell densities) will spontaneously produce TGF- $\beta$ ; (ii) hVSMC cultures from cells prepared by enzyme dispersal spontaneously produce substantial amounts of TGF- $\beta$  in culture (Kirschenlohr et al., Am. J. Physiol., 265, C571 (1993)) and therefore cannot be used for screening; and (iii) the sensitivity of rVSMCs and hVSMCs to agents which induce the cells to produce TGF- $\beta$  differs by up to 100-fold.

In screening for agents likely to be effective for clinical purposes, it is therefore necessary to use hVSMCs to determine both potency and the therapeutic window between effective concentrations and toxic concentrations for human cells. Candidate agents which pass the *in vitro* cell culture screens are then tested on one or more mouse models of lipid lesion formation. Efficacy of

candidate agents is tested by the protocols described in Example 7 for C57B16 mice and mice expressing the human apo(a) transgene that are fed a high fat diet, and also in apoE knockout mice fed a normal diet. Another animal model useful in screening agents is the cholesterol-fed Watanabe rabbit. Finally, small scale, pilot studies on candidate molecules are tested in patient groups with clinically significant coronary artery disease for the ability of the drug to increase circulating concentrations of active TGF- $\beta$  or to activate latent forms of TGF- $\beta$ .

The invention will be better understood by making reference to the following specific examples.

10

### EXAMPLE 1

#### Impact of Tamoxifen on Vascular Smooth Muscle Cells and the Relationship thereof to TGF-Beta Production and Activation

Cell culture, DNA synthesis assay and cell counting. Rat vascular smooth muscle cells were cultured after enzymatic dispersion of the aortic media from 12-17 week old Wistar rats as described in Grainger et al., Biochem. J. 277: 145-151, 1991. When the cells reached confluence (after about 6 days) the cells were released with trypsin/EDTA (available from Gibco) and diluted 1:2 in Dulbecco's modification of Eagle's medium (DMEM; available from ICN/Flow) supplemented with 100 U/ml penicillin and 10% fetal calf serum (FCS). The cells were then replated on tissue culture plastic (available from ICN/Flow) at approximately  $1 \times 10^4$  cells/cm<sup>2</sup>. The cells were subcultured repeatedly in this way when confluence was attained (about every 4 days), and the cells were used between passages 6 and 12.

Rat adventitial fibroblasts were cultured as described in Grainger et al., Biochem. J. 283: 403-408, 1992. Briefly, the aortae were treated with collagenase (3 mg/ml) for 30 minutes at 37°C. The tunica adventitia was stripped away from the media. The adventitia was dispersed for 2 hours in elastase (1 mg/ml) and collagenase (3 mg/ml) dissolved in medium M199 (available from ICN/Flow). The cells were then spun out (900 x g, 3 minutes), resuspended in DMEM + 10% FCS and plated out at  $8 \times 10^4$  cells/cm<sup>2</sup> on tissue culture plastic. When the cells reached confluence (after about 10 days), they

were subcultured as described for vascular smooth muscle cells. Adventitial fibroblasts were subcultured every 3 days at 1:3 dilution and used between passages 3 and 9.

DNA synthesis was assayed by [ $^3\text{H}$ ]-thymidine incorporation as described in Grainger et al., Biochem. J., 277:145-151, 1991. Vascular smooth muscle cells were subcultured, grown in DMEM + 10% FCS for 24 hours, made quiescent in serum-free DMEM for 48 hours and restimulated with 10% FCS at "0" hours. [ $^3\text{H}$ ]-thymidine (5 microcuries/ml; available from Amersham International) was added 12 hours after restimulation and the cells were harvested after 24 hours. DNA synthesis by adventitial fibroblasts was determined similarly, except that the cells were made quiescent in serum-free DMEM for 24 hours.

Cells were prepared for counting by hemocytometer from triplicate culture dishes as described in Grainger et al., Biochem. J., 277:145-151, 1991. Cells were also counted by direct microscopic observation of gridded culture dishes. The grids were scored into the plastic on the inner surface, so that the cells could not migrate into or out of the area being counted during the experiment. Cells in each of four squares in two separate wells were counted at each time point. All cell counting experiments were repeated on at least three separate cultures.

A stock solution of tamoxifen (5 mM; available from ICI Pharmaceuticals) was made up in 10% ethanol (EtOH) and diluted in DMEM and 10% FCS to give the final concentration. The effects of each tamoxifen concentration were compared with the effects observed in control wells containing the same final concentration of the ethanol vehicle. Recombinant TGF-beta (available from Amersham International) was dissolved in 25 mM Tris/Cl to give a 5 microgram/ml stock solution and sterile filtered through a Spinex Tube (such as a Centrex Disposable Microfilter Unit available from Rainin Instrument Company, Inc., Woburn, MA). Neutralizing antiserum to TGF-beta (BDA19; available from R & D Systems) was reconstituted in sterile MilliQ water (available from Millipore Corporation, Bedford, MA). At

10 micrograms/ml, this antibody completely abolished the activity of 10 ng/ml recombinant TGF-beta on subcultured (8th passage) vascular smooth muscle cells.

Assays for TGF-Beta. The TGF-beta activity present in medium  
5 conditioned on various cells was determined by DNA synthesis assay on mink lung endothelial (MvLu) cells; a modification of the assay described in Danielpour et al., J. Cell. Physiol., 138: 79-83, 1989. MvLu cells were subcultured at 1:5 dilution in DMEM + 10% FCS. After 24 hours, the medium was replaced with the conditioned medium to be tested in the absence or  
10 presence of the neutralizing antiserum to TGF-beta at 10 micrograms/ml. DNA synthesis during a 1 hour pulse of [<sup>3</sup>H]-thymidine (5 microcuries/ml) was determined 23 hours after addition of the test medium. TGF-beta activity was calculated as the proportion of the inhibition of DNA synthesis which was reversed in the presence of neutralizing antibody, using a standard curve to  
15 convert the inhibition values into quantities of TGF-beta. The TGF-beta standards and conditioned media both contained 10% FCS in DMEM.

The total latent and active TGF-beta present was determined by a sandwich ELISA (see Example 8). Maxisorb 96-well ELISA plates (available from Gibco) were coated with neutralizing antiserum against TGF-beta (BDA19;  
20 available from R & D Systems) at 2 micrograms/cm<sup>2</sup> in phosphate buffered saline (PBS) overnight at room temperature. The plates were washed between each step with tris-buffered saline containing 0.1% Triton X-100 (available from Sigma Chemical Company). The plates were incubated with samples for 2 hours, with a second antibody to TGF-beta (BDA5; available from R & D  
25 Systems) at 0.1 micrograms/ml for 2 hours, with anti-rabbit IgG peroxidase-conjugated antibody (available from Sigma Chemical Co.) for 1 hour, and with the chromogenic substrate o-phenylenediamine (Sigma), made up according to manufacturer's instructions, for 15 minutes. Absorbances at 492 nm were converted into quantities of TGF-beta protein using a standard curve. Both  
30 conditioned media and standards were assayed in the presence of 10% FCS in

DMEM. This assay was linear for TGF-beta concentrations in the range from 0.1 ng/ml to 20 ng/ml in the presence of 10% FCS in DMEM.

RNA Preparation and Northern Analysis. Total cytoplasmic RNA was isolated from cultured vascular smooth muscle cells as described in Kemp et al., *Biochem. J.*, **277**: 285-288, 1991. Northern analysis was performed by electrophoresis of total cytoplasmic RNA in 1.5% agarose gels in a buffer containing 2.2 M formaldehyde, 20 mM 3-(N-morpholino)propanesulfonic acid, 1 mM EDTA, 5 mM sodium acetate and 0.5 micrograms/ml ethidium bromide. The integrity of the RNA was checked by visualizing the gel under UV illumination prior to transfer onto Hybond N (available from Pharmacia LKB) as specified by the manufacturer. Filters were hybridized as described in Kemp et al., *Biochem. J.*, **277**: 285-288, 1991, using a [<sup>32</sup>P]-oligolabeled mouse TGF-beta probe corresponding to amino acids 68-228 in the precursor region of the TGF-beta polypeptide as set forth in Millan et al., *Development*, **111**: 131-144.

Results. Vascular smooth muscle cells from the aorta of adult rats proliferate with a cell cycle time of approximately 35 hours in DMEM + 10% FCS (see, for example, Grainger et al., *Biochem. J.*, **277**: 145-151, 1991). Addition of tamoxifen decreased the rate of proliferation with maximal inhibition at concentrations above 33 micromolar. 50 micromolar tamoxifen concentrations produced an increase in cell number (96 hours following the addition of serum) that was reduced by 66% +/- 5.2% (n=3). The slower rate of proliferation was hypothesized to stem from a complete blockage of proliferation for a proportion of the vascular smooth muscle cells or from an increase in the cell cycle time of all of the cells. To distinguish between these possibilities, the proportion of the cells passing through M phase and the time course of entry into cell division were determined.

Quiescent vascular smooth muscle cells were stimulated with DMEM + 10% FCS in the absence or presence of 33 micromolar tamoxifen, with the cell number being determined at 8 hour intervals by time lapse photomicroscopy. In the presence of ethanol vehicle alone, more than 95% of the vascular smooth muscle cells had divided by 40 hours, whereas there was no significant increase



in cell number in the presence of tamoxifen until after 48 hours. By 64 hours, however, more than 90% of the cells had divided in the presence of tamoxifen. The time taken for 50% of the cells to divide after stimulation by serum was increased from 35  $\pm$  3 hours (n=7) to 54  $\pm$  2 hours (n=3) by 33 micromolar tamoxifen. Since tamoxifen did not significantly reduce the proportion of cells completing the cell cycle and dividing, inhibition of vascular smooth muscle cells caused by tamoxifen appears to be the result of an increase in the cell cycle time of nearly all (>90%) of the proliferating cells.

To determine whether tamoxifen increased the duration of the cell cycle of vascular smooth muscle cells by increasing the duration of the G<sub>0</sub> to S phase, the effect of tamoxifen on entry into DNA synthesis was analyzed. Tamoxifen at concentrations up to 50 micromolar did not significantly affect the time course or the proportion of cells entering DNA synthesis following serum stimulation of quiescent vascular smooth muscle cells (DNA synthesis between 12 hours and 24 hours after stimulation was measured by [<sup>3</sup>H]-thymidine incorporation: control at 17614  $\pm$  1714 cpm; 10 micromolar tamoxifen at 16898  $\pm$  3417 cpm; and 50 micromolar tamoxifen at 18002  $\pm$  4167 cpm). Since the duration of S phase is approximately 12 hours (unpublished data), tamoxifen does not appear to have significantly impacted the time course of entry into DNA synthesis. These results therefore imply that tamoxifen decreases the rate of proliferation of serum-stimulated vascular smooth muscle cells by increasing the time taken to traverse the G<sub>2</sub> to M phase of the cell cycle.

Based upon these results, it appeared that tamoxifen exhibited effects similar to those previously described for TGF-beta (see, for example, Assoian et al., J. Cell. Biol., 109: 441-448, 1986) with respect to proliferation of subcultured vascular smooth muscle cells in the presence of serum. Tamoxifen is known to induce TGF-beta activity in cultures of breast carcinoma cell lines as described, for example, in Knabbe, et al., Cell, 48: 417-425, 1987.

Consequently, experimentation was conducted to determine whether tamoxifen decreased the rate of proliferation of vascular smooth muscle cells by inducing TGF-beta activity. When quiescent vascular smooth muscle cells were

stimulated with 10% FCS in the presence of 50 micromolar tamoxifen and 10 micrograms/ml neutralizing antiserum against TGF-beta, the cells proliferated at the same rate as control cells in the presence of ethanol vehicle alone.

To confirm that the vascular smooth muscle cells produced TGF-beta in response to tamoxifen, such cells were treated with tamoxifen for 96 hours in the presence of 10% FCS. The conditioned medium was then collected and TGF-beta activity was determined by the modified mink lung epithelial (MvLu) cell assay described above. Tamoxifen increased the TGF-beta activity in the medium by > 50-fold. Addition of tamoxifen (50 micromolar) in fresh DMEM + 10% FCS to the MvLu cells had no effect on DNA synthesis, demonstrating that tamoxifen did not induce production of active TGF-beta by the MvLu cells.

TGF-beta is produced as a latent propeptide which can be activated outside the cell by proteases such as plasmin. To determine whether tamoxifen increased TGF-beta activity by promoting the activation of latent TGF-beta or by stimulating the production of the latent propeptide which was subsequently activated, the total latent plus active TGF-beta present in the conditioned medium was determined by sandwich ELISA as described above. After 96 hours in the presence of tamoxifen (50 micromolar), the total TGF-beta protein present was increased by approximately 4-fold. Furthermore, the proportion of the TGF-beta present in active form was increased from < 5% in the medium conditioned on vascular smooth muscle cells in the presence of ethanol vehicle alone to approximately 35% in the medium conditioned on cells treated with tamoxifen. Thus, tamoxifen appears to increase TGF-beta activity in cultures of rat vascular smooth muscle cells by stimulating the production of latent TGF-beta and increasing the proportion of the total TGF-beta which has been activated.

Heparin increases TGF-beta activity in medium conditioned on vascular smooth muscle cells (unpublished data). The mechanism of action of heparin in this regard appears to involve the release of TGF-beta from inactive complexes present in serum, because pretreatment of serum with heparin immobilized on agarose beads is as effective as direct addition of free heparin to the cells. To determine whether tamoxifen acts to release TGF-beta from sequestered

complexes in serum which are not immunoreactive in the ELISA assay, 10% FCS + DMEM was treated with 50 micromolar tamoxifen for 96 hours at 37°C in the absence of cells. Medium treated in this way contained similar levels of TGF-beta protein and activity to untreated medium. It appears, therefore, that  
5 tamoxifen, unlike heparin, does not act by releasing TGF-beta from inactive complexes present in serum.

The content of TGF-beta mRNA was also analyzed by Northern analysis at various time points after addition of tamoxifen. Subcultured rat vascular smooth muscle cells (6th passage in exponential growth) in the absence or  
10 presence of ethanol vehicle alone contain very little mRNA for TGF-beta. By 24 hours after addition of tamoxifen (10 micromolar), TGF-beta mRNA was increased approximately 10-fold.

Although TGF-beta decreases the rate of proliferation of vascular smooth muscle cells, it does not affect the rate of proliferation of fibroblasts. Tamoxifen  
15 at concentrations of up to 50 micromolar did not reduce the rate of proliferation of subcultured adventitial fibroblasts. Tamoxifen is therefore a selective inhibitor of vascular smooth muscle proliferation with an ED<sub>50</sub> at least 10-fold lower for vascular smooth muscle cells than for adventitial fibroblasts.

## EXAMPLE 2

### 20 Heparin Effect on VSMC Proliferation and Differentiation

Heparins. An unfractionated, high molecular weight, anticoagulant pig mucosal heparin, fragments of heparin devoid of anticoagulant activity, and fragments of heparin with anticoagulant activity were tested. In addition, heparin coupled to agarose beads (Sigma Chemical Co., St. Louis, MO) was  
25 examined (see also Grainger et al., Cardiovascular Res. 27:2238-47, 1993).

Effect on proliferation. Freshly dispersed rat VSMC, prepared as in Example 1, were cultured in medium containing serum (as in Example 1) in the presence or absence of heparin. The cells were counted at intervals. Depending on the heparin used, the increase in cell number at 144 hours (when control cells  
30 enter stationary phase) was reduced by between 27±4.2% and 76±3.2% ( $p < 0.0005$  compared with cell number in control wells for all heparins tested).

Although the effects of the heparins at 100 µg/ml were similar, there was a trend to greater effectiveness with increasing molecular size. The four heparins of 20 kD or above inhibited proliferation by 60-76%, and the four heparins of 12.6-3 kD inhibited proliferation by 27-45%.

- 5        Entry into cell cycle phases. Heparin had no effect on the entry of cells into S phase, as determined by growing the cells in the presence of 10 µM bromodeoxyuridine from 0-72 hours. Similar results were obtained when the cells were pulse-labeled with [<sup>3</sup>H]-thymidine.

- 10        The proportion of cells completing mitosis in the presence or absence of heparin was determined. Defined fields of cells were photographed at eight hour intervals by time lapse microscopy of gridded culture dishes. The grids were scored into the plastic on the inner surface so that the cells could not migrate into or out of the area being counted. In the absence of heparin, 92±1% of primary cells divided by 60 hours, but there was no detectable cell division in the  
15        presence of heparin until 72 hours. By 88 hours, however, 96±2% of the cells had divided in the presence of heparin. In the presence or absence of heparin, the time to complete mitosis was less than 3 hours. The total cell cycle times in the presence and absence of heparin were determined. The data showed that the major effect of heparin was to extend selectively the duration of G<sub>2</sub> to M phase  
20        of the cell cycle.

The concentration of heparin required to inhibit S phase entry decreased as the serum concentration was reduced. This observation is consistent with the removal by heparin of components of serum required for progression to S phase.

- 25        Heparin and TGF-beta. To determine whether TGF-beta mediated the effects of heparin, anti-TGF-beta antibody (10 µg/ml; R&D Systems) was added. Anti-TGF-beta antibody alone had no effect on VSMC proliferation stimulated by 10% FCS. This antibody completely reversed the inhibition of VSMC proliferation observed when cells were incubated in the presence of heparin. Heparin coupled to agarose beads at an extracellular concentration of 100 µg/ml  
30        was as effective as free heparin (100 µg/ml) at inhibiting VSMC proliferation. Agarose beads alone at the same concentration had no effect. These results are

consistent with extracellular action of heparin on VSMC to inhibit proliferation. Further cell cycle studies indicated that heparin must be present within the first 12 hours of G<sub>1</sub> to inhibit VSMC proliferation.

Heparin and smooth muscle-specific myosin heavy chain expression.

5 Previous studies demonstrated that primary VSMC in culture lose both the 204 kD (SM-1) and the 200 kD (SM-2) isoforms of SM-MHC, whether the VSMC are cultured in serum or in serum-free medium onto fibronectin. In primary cultures stimulated by serum, 100 µg/ml heparin substantially inhibited the loss of both SM-1 and SM-2 proteins in all cells, as assayed by direct  
10 immunoperoxidase staining or Western blotting (Cell Tissues Res. 257:1137-39, 1989; Biochem. J. 277:145-51, 1991). If the cells were plated in serum-free medium onto fibronectin, the normal loss of SM-1 and MS-2 proteins was unaffected by the presence of heparin. The effect of heparin in preventing the de-differentiation of primary VSMC in serum was completely reversed by the  
15 addition of anti-TGF-beta antibody (10 µg/ml), indicating that this heparin effect was also mediated by TGF-beta-like activity. Although heparin prevented the loss of smooth muscle-specific myosin heavy chain from primary VSMC in the presence of serum, it did not promote its reexpression. Moreover, heparin did not promote reexpression of SM-MHC in subcultured cells that exhibit very low  
20 levels of this protein. Thus, the effects of heparin and TGF-beta on the expression of SM-MHC in primary VSMC are similar.

**EXAMPLE 3**

Comparison of Enzyme-Dispersed and

Explant-Derived Human VSMC

25 Materials. Collagenase (C-0130), elastase (E-0258), anti-rabbit IgG peroxidase-conjugated antibody, the chromogenic substrate orthophenylenediamine, and streptomycin sulfate were obtained from Sigma. Tamoxifen (free base) was purchased from Aldrich. Dulbecco's modified Eagle's Medium (D-MEM) and medium M199 were purchased from Flow Laboratories.  
30 6-[<sup>3</sup>H]-thymidine and the cell proliferation kit were obtained from Amersham International. Anti-TGF-beta antibodies (BDA19 and BDA47) were purchased

from R&D Systems. EGF, PDGF-AA and PDGF-BB were obtained from Bachem, and were dissolved in filter-sterilized 25 mM Tris-HCl, pH 7.5, containing 1% fatty acid-free bovine serum albumin (BSA). Basic fibroblast growth factor and insulin-like growth factor 1 (N-mer) were obtained from Bachem and dissolved in sterile MilliQ water. Angiotensin II and endothelin 1 were obtained from Sigma and dissolved in sterile MilliQ water. TGF-beta (0.5 µg, lyophilized solid) was purchased from Peninsula, dissolved in 5 mM HCl to yield a 5 µg/ml stock, and diluted with PBS + 0.2% BSA.

Human aortic VSMC cultures. Adult human VSMC were obtained from 6 transplant donors (either sex, age range from 3 to 54 years) using the enzyme dispersal or explant technique. In one case, the same donor (a 24 year old male) was used to establish both an enzyme-dispersed (ED) and explant-derived (EX) cell culture. Prior to enzyme-dispersion or explanting treatment, human aortas were obtained within 18 hours of death. The endothelium layer was removed with a scalpel blade and strips of smooth muscle cells (tunica media) were removed with forceps and chopped into small pieces (1 mm<sup>3</sup>).

ED Cultures. The aortic pieces were washed once with serum-free Hanks Balanced Salt Solution, then enzyme-dispersed with collagenase and elastase, as described in Example 1. The cells were plated at an initial density of  $1.5 \times 10^5$  cells/cm<sup>2</sup> and incubated in a humidified atmosphere at 37°C in 5% CO<sub>2</sub> in air. The cells were subcultured every 6-7 days (at stationary phase) by releasing them with trypsin/EDTA and diluting them 1:1.5 in D-MEM + 10% FCS. Subcultured ED cells were cultured with D-MEM + 20% FCS 24 h after plating, and thereafter at 48 hour intervals.

EX Cultures. The aortic pieces were washed once with D-MEM + 10% FCS, resuspended in a small volume of fresh D-MEM + 10% FCS, and transferred to culture flasks or Petri dishes. The pieces were allowed to sediment onto the plastic and were evenly distributed ( $\approx 4$  pieces/cm<sup>2</sup>). Cells started to grow out from the explants after 3-7 days in culture. The aortic pieces were removed during the third week in culture, and the cells adhering to the plastic were allowed to grow to confluence for a further week. The cells were then

subcultured every 4-5 days by releasing them with trypsin/EDTA and diluting them 1:2 in D-MEM + 10% FCS. Subcultured cells were incubated with fresh D-MEM + 20% FCS as described for ED cultures.

ED and EX subcultures were used between passage 5-20.

- 5 Cell counting, DNA synthesis assays and assays for total and active TGF-beta were performed as described in Examples 1 and 8.

### Results.

- 10 ED and EX cultures prepared from the aorta of a single individual displayed distinct morphologies and growth characteristics. The EX culture proliferated much more rapidly than the ED culture. After 6 weeks of subculturing the ED and EX culture whenever confluence was attained, the total yield of cells was 4 fold higher per gram wet weight of aorta in the EX culture  
15 than the ED culture. The ED culture had a longer population doubling time in D-MEM + 20% FCS ( $71 \pm 5$  hours) than the EX culture ( $35 \pm 2$  hours).

- The VSMC in the EX culture were spindle-shaped and grew to confluence with a characteristic "hills and valleys" pattern at confluence. The EX culture VSMC reached stationary phase at a high saturation density ( $2.0 - 4.0$   
20  $\times 10^4$  cells/cm<sup>2</sup>). In contrast, the VSMC in the ED culture had a stellate morphology with numerous long cytoplasmic projections. They reached stationary phase at a low saturation density ( $0.7 - 2.0 \times 10^4$  cells/cm<sup>2</sup>) without reaching monolayer coverage of the substrate. The VSMC in the ED culture contained high levels of both SM-MHC and  $\alpha$ -actin, while the VSMC in the EX  
25 culture contained much lower levels of both of these protein markers.

- The longer population doubling time of human ED cultures compared to ED cultures from the rat aorta is due to autocrine production of active TGF-beta. These human ED cultures produced  $15.2 \pm 1.6$  ng/ml total TGF-beta protein, of which  $64 \pm 12\%$  was in the active form. In contrast, the human EX cultures did  
30 not produce detectable amounts of TGF-beta. Medium conditioned for 48 hours on EX cultures during exponential growth contained  $<1$  ng/ml total TGF-beta.

When TGF-beta production was compared using ED and EX cultures obtained from the same donor, the ED culture produced 8.5 ng/ml total TGF-beta, of which 57% was in the active form. The corresponding EX culture produced <1 ng/ml total TGF-beta protein.

5        Exogenous TGF-beta (10 ng/ml) was added to EX cultures 24 hours after subculturing and cell number was determined at 24 hour intervals. After 96 hours in the presence of exogenous TGF-beta, the increase in cell number was inhibited by  $34 \pm 2\%$ . The population doubling time of the EX cultures increased from  $32 \pm 1$  hour to  $42 \pm 3$  hours in the presence of exogenous TGF-beta.

10       Because the addition of exogenous TGF-beta extended the population doubling time of EX cultures by less than 12 hours, TGF-beta activity alone cannot account for the difference in population doubling time between the ED and EX cultures. Therefore, the fraction of cells that entered DNA synthesis in a 6 day period was compared using bromodeoxyuridine incorporation with a cell  
15       proliferation kit. The proportion of EX culture nuclei demonstrating bromodeoxyuridine incorporation after a 6 day pulse was  $86 \pm 4\%$ , but for ED culture cells was  $48 \pm 4\%$ . Therefore, the population doubling time of ED cultures was further increased over that of EX cultures, because less of the ED cells than the EX cells were cycling in the presence of D-MEM + 20% FCS.

20       Tamoxifen (TMX) inhibits proliferation of rat ED VSMC by inducing TGF-beta production with a half-maximal inhibition of proliferation at  $2.5 \mu\text{M}$  TMX. Because human ED cultures already produce autocrine TGF-beta, the addition of TMX would not be expected to reduce the rate of VSMC proliferation further. To confirm this prediction, various concentrations of TMX  
25       (1 nM to  $100 \mu\text{M}$ ) or ethanol vehicle only (20 ppm to 0.2%) were added to the human VSMC for 96 hours, and the cell number was determined by cell counting. Concentrations of TMX  $> 33 \mu\text{M}$  caused cell death, but concentrations below  $10 \mu\text{M}$  did not affect the rate of proliferation.

EX cultures of human VSMC did not produce autocrine TGF-beta, so  
30       TMX would be predicted to inhibit VSMC proliferation. Concentrations of  $> 33 \mu\text{M}$  TMX caused cell death in human EX cultures, as observed with human



ED cultures. The half-maximal inhibitory dose for EX cultures was 30-100 nM TMX. At 5  $\mu$ M TMX, the increase in cell number in human EX cultures was inhibited  $33 \pm 8\%$ .

To confirm these observations, quiescent EX cultures were restimulated and cultured for 96 hours in D-MEM + 20% FCS containing TMX (0.5  $\mu$ M) in the presence or absence of anti-TGF-beta antibody (25  $\mu$ g/ml). The increase in cell number in the presence of TMX alone was inhibited by  $27 \pm 2\%$ , as compared to control cells incubated with ethanol vehicle alone. The presence of anti-TGF-beta antibody completely reversed the inhibition of proliferation due to TMX.

10 ELISA assays for TGF-beta confirmed that medium conditioned on human EX cultures in the presence of 5  $\mu$ M TMX contained  $6.0 \pm 2.0$  ng/ml total TGF-beta protein, of which  $55 \pm 5\%$  was activated.

The effect of heparin on proliferation of human ED and EX cultures was examined. Heparin IC86-1771, known to inhibit proliferation of rat ED VSMC  
15 by releasing a TGF-beta-like activity from serum, partially inhibited the proliferation of human EX cultures, but not ED cultures. At 100  $\mu$ g/ml and at 48 hours after addition, heparin inhibited the increase in cell number in EX cultures by  $51 \pm 10\%$ ; at 96 hours after addition, by  $71 \pm 15\%$ . In ED cultures at 96 hours after addition of 100  $\mu$ g/ml heparin, the increase in cell number was  
20 inhibited by  $8 \pm 5\%$ . Anti-TGF-beta antibody did not abolish the ability of heparin to inhibit the proliferation of human EX cultured VSMC. Therefore, human EX VSMC may release more TGF-beta from 20% FCS than could be neutralized by added antibody, or heparin affected TGF-beta DNA synthesis as well as TGF-beta activation at the heparin concentrations tested.

25 The effect of mitogens on the entry of ED and EX cells into DNA synthesis was examined. Quiescent ED and EX VSMC were restimulated with either 20% FCS or 100 ng/ml PDGF-BB in D-MEM, and entry into DNA synthesis was monitored during successive 8 hour pulses using [ $^3$ H]thymidine. EX cells entered DNA synthesis in response to both mitogenic stimuli more  
30 rapidly than ED cells. The EX cells reached peak rate of DNA synthesis in

response to FCS 16-24 hours after stimulation. The ED cells reached peak rate of DNA synthesis 24-32 hours after mitogenic stimulation.

Quiescent EX cells were then exposed to various mitogens, and stimulation of DNA synthesis was determined by incorporation of [<sup>3</sup>H]thymidine 5 16-32 hours after stimulation. DNA synthesis was stimulated by 20% FCS by 8.0±1.5 fold, compared to control cells that remained in serum-free D-MEM throughout. PDGF-BB and PDGF-AA caused a ≈ 3.0 fold stimulation of DNA synthesis. Insulin-like growth factor (IGF-1; 25 ng/ml) provided a 1.2 fold stimulation. However, epidermal growth factor (EGF; 100 ng/ml), basic 10 fibroblast growth factor (bFGF; 100 ng/ml), TGF-beta (10 ng/ml), angiotensin II (AII; 100 nM) and endothelin-1 (ET-1; 100 nM) did not significantly stimulate DNA synthesis.

Quiescent ED cells were exposed to various mitogens, and stimulation of DNA synthesis was determined by [<sup>3</sup>H]thymidine incorporation 16-40 hours 15 after stimulation. DNA synthesis was stimulated by 20% FCS by 25±6 fold, compared to control cells that remained in serum-free D-MEM throughout. PDGF-BB stimulated ≈3.0 fold, but PDGF-AA stimulated only 2.0 fold. The latter response was also variable (1 of 3 cultures did not respond to PDGF-AA), in contrast to the stimulation of EX VSMC. IGF-1 and EGF stimulated DNA 20 synthesis 1.3 fold, and bFGF, TGF-beta, AII and ET-1 did not stimulate DNA synthesis.

#### EXAMPLE 4

##### TGF-beta and Transgenic apo(a) Mice

Apo(a) mice. Human apo(a) has been expressed in transgenic mice 25 (Nature 360:670-72,1992), a species that normally lacks apo(a). These mice were used to study whether inhibition of TGF-beta activation, resulting in enhanced VSMC proliferation, represents a key-step in atherogenesis.

Apo(a) transgenic mice, when fed a lipid-rich diet, develop vascular lesions similar to the fatty streak lesions in early human atherosclerosis. 30 Immunoperoxidase labeling showed that apo(a) accumulated in the vessel wall at strongly staining focal regions in the luminal surface of the vessel. This

phenomenon was studied using the more sensitive technique of immunofluorescence labeling.

Briefly, transgenic apo(a) mice, confirmed for the presence of the apo(a) gene by Southern blotting, and normal litter mates were obtained by continued crossing of transgenic mice with C57/B16 x SJL hybrids. The heart and attached aorta were dissected out, immediately frozen in liquid nitrogen, embedded, and 6  $\mu$ m frozen sections were prepared. The sections were fixed in ice-cold acetone for 90 seconds and stored at -20°C until used. All fluorescent labeling procedures were performed at 4°C. For apo(a) immunolabeling, sections were incubated with 3% BSA in Tris-buffered saline (TBS) for 30 minutes, then with sheep anti-human Lp(a) antibody that had been adsorbed against human plasminogen diluted 1:1000 in TBS containing 3% BSA. The anti-human Lp(a) antibody had no detectable cross-reactivity with mouse plasminogen. The bound primary antibody was detected using fluorescein-conjugated rabbit anti-sheep IgG diluted 1:80 in TBS containing 3% BSA, and visualized by fluorescence microscopy at 400x magnification ( $\lambda_{exc}=440\text{nm}$ ;  $\lambda_{em}=510\text{nm}$ ); photomicrographs were taken with 5 second exposures (ASA 1600). The tissue sections were indistinguishable whether the mice were fed a normal diet (Techlad, Madison, Wisconsin; 4% mouse/rat chow) or a lipid-rich diet containing 1.25% cholesterol, 7.5% saturated fat as cocoa butter, 7.5% casein and 0.5% sodium chelate.

Immunofluorescence labeling for apo(a) showed strongly labeled foci of apo(a) in the luminal surface of the aortic wall, but apo(a) was also labeled at a substantially lower intensity throughout the media of the vessel. No apo(a) labeling was detected in the aortic sections from the normal litter mate mice. The serum concentration of apo(a) in the transgenic mice was  $3.8 \pm 1.2$  mg/dl. Analysis of human arteries and of mice injected with radiolabeled apo(a) showed that plasma-derived apo(a) penetrates the vessel wall. *In situ* hybridization suggested that little, if any, apo(a) in the vessel wall of the apo(a) mice was derived from local synthesis.

Total and activated plasminogen. Activation of plasminogen in the aortic wall was assayed using the specific inhibitor,  $\alpha 2$ -antiplasmin ( $\alpha 2$ -AP), which forms a stable covalent conjugate with active plasmin, but does not bind covalently to plasminogen, apo(a) or other proteins in the vessel wall. Briefly,  $\alpha 2$ -AP (Sigma) was labeled with either fluorescein isothiocyanate (Sigma) or trimethylrhodamine isothiocyanate (*Experimentia* 16:430, 1960), and separated from unincorporated label by two gel filtrations on Sephadex G25.

For determination of activated plasminogen, sections were incubated for 16 hours with  $\alpha 2$ -AP-FITC (1  $\mu$ g/ml) and washed. For determination of total plasminogen, the sections were incubated with  $\alpha 2$ -AP-FITC, as above, washed thoroughly in TBS containing 0.2% Nonidet-P40 (NP-40) and 300 mM NaCl (wash buffer), and then incubated with 1 mg/ml recombinant human tissue plasminogen activator (rTPA) in TBS for 3 hours to activate the plasminogen. The sections were washed, incubated for 16 hours with  $\alpha 2$ -AP-TRITC (1  $\mu$ g/ml), then washed thoroughly in wash buffer, followed by TBS. Bound labeled  $\alpha 2$ -AP was visualized by fluorescence microscopy at 400x magnification ( $\lambda_{exc}=440\text{nm}$ ;  $\lambda_{em}=510\text{nm}$  for FITC label;  $\lambda_{exc}=490\text{nm}$ ;  $\lambda_{em}=580\text{nm}$  for TRITC label). The low level of background autofluorescence from the acetone-fixed sections was subtracted for each section from the fluorescence of the label. There were no significant differences in the autofluorescence intensity either between sections from the same mouse aorta, or between normal litter mate aortic sections and those from transgenic apo(a) mice. Photomicrographs of bound  $\alpha 2$ -AP-FITC to detect active plasmin were exposed for 10 seconds, and of bound  $\alpha 2$ -AP-TRITC to detect plasminogen were exposed for 1 second (1600 ASA).

Quantitation of fluorescence. A Magiscan image analysis system (Joyce-Loebl) with extended linear range TV camera (Photonic Science) attached to a Nikon Diaphor inverted fluorescence microscope was used to quantitate the fluorescence. The gain control on the photomultiplier was set so that the average pixel value over the area of the vessel wall was between 2-5% of full scale. For each section, four fields of aortic wall were selected randomly under phase contrast (400x magnification), and separate fluorescence images were captured

using filters for fluorescein and trimethylrhodamine. For TGF-beta and plasminogen/plasmin, the average pixel value for the fluorescence intensity over the whole area of the vessel media was calculated, and the mean for the four sections from each mouse (i.e., 16 fields of view) was computed. For osteopontin, the vessel media was only partly labeled, and only pixels with intensity values >5% of full scale were included in the calculation of average pixel value. The number of pixels ( $\times 10^{-2}$ ) above the threshold is shown as the area labeled for osteopontin.

The  $\alpha 2$ -AP-FITC was detected in aortic sections of both the normal and apo(a) mice, predominantly associated with the elastic laminae of the vessels. Quantitation of the fluorescent label showed approximately 3 fold less active plasmin in the vessel wall of the apo(a) mice than in the normal mice, regardless of whether the mice had been fed a lipid-rich or normal diet, as shown in Table 1.

TABLE 1

Quantitative fluorescent data

	Normal Mice		Transgenic apo(a) Mice	
	Normal Diet	Lipid-Rich	Normal Diet	Lipid-Rich
TGF- $\beta$				
Total	112 $\pm$ 7	95 $\pm$ 12	115 $\pm$ 1	109 $\pm$ 6
% Active	90 $\pm$ 6	90 $\pm$ 5	36 $\pm$ 3*	46 $\pm$ 8*
Plasminogen				
Total	702 $\pm$ 47	748 $\pm$ 95	789 $\pm$ 121	688 $\pm$ 133
% Active	6.3 $\pm$ 1.3	6.1 $\pm$ 0.6	1.7 $\pm$ 0.7*	1.9 $\pm$ 1.2*
Osteopontin				
Total	1.4 $\pm$ 0.8	0.4 $\pm$ 0.1	32.3 $\pm$ 4.4*	12.6 $\pm$ 2.1**
Area	0.7 $\pm$ 0.9	1.2 $\pm$ 1.6	80.3 $\pm$ 0.0*	103 $\pm$ 31.7**

\* p < 0.05 for apo(a) mice compared with normal litter mate mice

+ p < 0.05 for apo(a) mice on a lipid-rich diet compared with apo(a) mice on a normal diet (Student's unpaired t-test)

Control experiments demonstrated that the  $\alpha 2$ -AP-FITC bound only to active plasmin in the sections. No fluorescence was detected in aortic sections that were incubated with  $\alpha 2$ -AP-FITC in the presence of a large excess (1 mU) of exogenous active plasmin. Aortic sections were also incubated with  $\alpha 2$ -AP-FITC after treatment with the plasmin inhibitor, aprotinin (100  $\mu$ g/ml), and no fluorescence was detected, demonstrating that there was no interaction of the label with the sections in the absence of active plasmin.

To assay for plasminogen, active plasmin was first labeled with  $\alpha 2$ -AP-FITC, as described above, then the same sections were treated with rTPA to activate the plasminogen. The sections were relabeled for active plasminogen using  $\alpha 2$ -AP-TRITC. When the rt-PA was omitted, no further staining for active plasmin with  $\alpha 2$ -AP-TRITC was observed. Quantitation of the two fluorescent labels of active plasmin before and after activation of the plasminogen provides a measure of the total amount of plasminogen and of the proportion of plasminogen that was already activated in the sections (see Table 1). There was no significant difference in the total amounts of plasminogen in the sections from the apo(a) mice and normal mice. In the normal mice,  $\approx 6\%$  of the plasminogen was activated to plasmin, compared with only 2% in the apo(a) transgenic mice. Thus, apo(a) inhibits plasminogen activation.

TGF-beta. To determine whether the low plasmin concentration in the aortic wall of the apo(a) mice resulted in reduced activation of TGF-beta, immunofluorescent labels were used to quantitate active TGF-beta and total TGF-beta (active + latent). Briefly, sections prepared as described above were labeled for total TGF-beta for 2 hours with 25  $\mu$ g/ml of BDA47 (R&D Systems), a rabbit polyclonal antiserum to TGF-beta that detects isoforms 1 and 3 with equal sensitivity, but does not distinguish between latent and active TGF-beta. The sections were washed 3 times in TBS, and incubated with goat anti-rabbit IgG (Sigma; 1:50 dilution) conjugated with TRITC. Both antibodies were diluted in TBS containing 3% BSA. The same section was then washed 3 times in TBS and labeled for active TGF-beta with R2X (TGF-beta type II receptor extracellular domain, which recognizes the active form of isoforms 1 and 3 only)

that was conjugated with FITC, as described above. Sections were incubated for 16 hours, then washed 3 times in PBS. Bound label was visualized by fluorescence microscopy, as described above. Photomicrograph exposures were 5 seconds (1600 ASA). To calibrate the fluorescence intensities of the two labels, a solution containing various proportions of active TGF-beta (6 ng/ml of total TGF-beta) was spotted on gelatin-polylysine-coated slides and allowed to dry at room temperature. The protein spots were labeled for total and active TGF-beta, as described for the aortic sections, and the fluorescence intensity ratios (TRITC/FITC) were determined. False color images of the proportion of TGF-beta in the active form were computed from the fluorescence ratios of the aortic sections using the calibration.

TGF-beta was present throughout the aortic media, predominantly associated with the elastic laminae in both the normal and apo(a) mice. No fluorescent label was bound to the sections when the primary anti-TGF-beta antibody was omitted. Quantitation of the fluorescent label showed no significant difference in the total amount of TGF-beta present in the aortic wall of normal and apo(a) mice (see Table 1).

Active TGF-beta was assayed using a truncated extracellular domain of the type II TGF-beta receptor fused to glutathione-S-transferase (R2X) that had been FITC labeled. This label was detected in sections from both normal and apo(a) mice in association with the elastic laminae. In the presence of 100 mg/ml recombinant active TGF-beta-1, the binding of R2X-FITC to the sections was completely blocked. In addition, glutathione-S-transferase labeled with FITC did not detectably bind to aortic sections from either normal or apo(a) mice.

The TGF-beta present in the aortic wall from apo(a) mice was significantly less active than the TGF-beta in the aortic wall from normal mice, irrespective of whether the mice had been fed a lipid-rich diet or normal diet (see Table 1). Thus, TGF-beta activation in the aortic wall is significantly inhibited by the presence of apo(a). Moreover, activation of TGF-beta is most strongly inhibited at the sites of highest apo(a) accumulation. Therefore, changes in the

vessel wall that are a consequence of reduced TGF-beta activity will occur preferentially at the sites of focal apo(a) accumulation, but will not be dependent on the accumulation of lipid.

The mouse serum was also assayed for inhibition of TGF-beta activation by apo(a), using ELISAs for total and active TGF-beta (see Example 8). The total TGF-beta in the serum of apo(a) mice was  $14.4 \pm 4.7$  ng/ml; in normal mice it was  $14.2 \pm 3.5$  ng/ml. However, the proportion of total TGF-beta that was active in the serum of apo(a) mice was  $34 \pm 19\%$ , compared with  $92 \pm 12\%$  active TGF-beta in the serum of normal mice.

10        Osteopontin. Aortic sections were assayed for osteopontin, a marker of activated smooth muscle cells. Osteopontin was detected by incubating sections with monoclonal antibody MPIIB10, (National Institute of Health Developmental Studies Hybridoma Bank) at  $10 \mu\text{g/ml}$  in TBS containing 3% BSA for 16 hours. The sections were washed 3 times in TBS, and bound  
15        antibody was detected using goat anti-mouse IgG conjugated to fluorescein (Sigma F-2012; 1:50 dilution; 2 hours). Photomicrographs were obtained with 2.5 sec exposure time (ASA 1600).

Fluorescent labeling of osteopontin was detected in the aortic sections from apo(a) mice on either a lipid-rich or normal diet. Although a small increase  
20        in labeling for osteopontin was detected throughout the media of the aortae from transgenic apo(a) mice, very high levels of osteopontin labeling were co-localized with regions of focal apo(a) accumulation and very low TGF-beta activation. Treatment of apo(a) mice with bromodeoxyuridine for 24 hours before sacrifice showed no significant mitotic activity in the aortic media. Thus,  
25        in the absence of physical injury, replication rates in atheromatous plaques are low, reflecting the slow growth of the lesions. Areas of aortic sections from normal mice that showed high proportions of active TGF-beta did not show detectable labeling for osteopontin. The total intensity and area of osteopontin labeling in the normal mouse sections were also very low compared with the  
30        apo(a) mouse sections. Therefore, the presence of apo(a) induces osteopontin expression in VSMC in the aortic wall, similar to the changes that occur during



the development of vascular lesions, regardless of whether the mice are fed a lipid-rich or normal diet. Accumulation of lipid into the vessel wall under conditions where circulating lipid is elevated may be a consequence, rather than a cause, of the changes in VSMC activation marked by the expression of osteopontin. Previous studies have shown that activated VSMC in culture accumulate about 20 fold more lipid than contractile VSMC.

The results of these experiments link apo(a) to the inhibition of plasminogen and latent TGF-beta activation. The inhibition of TGF-beta activation likely contributes to the subsequent development of fatty lesions when apo(a) containing subjects (mice or human) are subject to a lipid-rich diet.

#### EXAMPLE 5

##### Tamoxifen Inhibits Migration and

##### Lipid Uptake in VSMC *in vitro* and in Transgenic Mice

Cell culture. Rat aortic VSMCs from 12-20 week old Wistar male rats were prepared by enzyme dispersion, as described in Example 1. The cultured cells were confirmed as >99% SMC by staining for SM-MHC, and proliferated with a cell cycle time of 36 h. Cells were passaged as described in Example 1, and were used either in primary culture or between passages 6-12.

Human aortic SMC from donors of either sex, aged 15-60, were prepared by explanting 1 mm<sup>3</sup> of medial tissue, as described in Example 3.

Migration. Migration was assayed using SMC grown to confluence on glass coverslips. A defined injury is performed on the confluent layer of cells, which are allowed to recover in D-MEM + 10% FCS for 24 hours.

Bromodeoxyuridine (10  $\mu$ M) is added between 18-24 hours, to label proliferating cells. Cells migrating past the boundary of the wound edge at 24 hours are detected by propidium iodide (PI) staining of the cell nuclei (500  $\mu$ M PI in PBS + 0.5% NP-40 for 30 min at room temperature). Cells that synthesized DNA were detected by antibody staining for bromodeoxyuridine using fluorescein-conjugated anti-bromodeoxyuridine antibodies. Migrating and proliferating cells in each field of view were simultaneously counted by image

analysis of the rhodamine emission from PI and fluorescein emission from bromodeoxyuridine.

Lipid uptake. Cells in 24 well plastic dishes were incubated with serum-free D-MEM for 24 hours or 1 hour at 37°C, then washed in PBS + 1% BSA at 4°C on ice for 30 minutes. Cells were incubated with <sup>125</sup>I-labeled LDL at various concentrations for 3 hours in the presence or absence of cold competitor LDL. The cells were washed six times with ice-cold PBS, lysed in 0.1 M NaOH or 0.1% SDS, and cell-associated counts of LDL were determined by gamma counting.

10 Apo(a) transgenic mice. Apo(a) [human 500 kD isoform] was expressed from the transferrin promotor in C57/B16 x SJL F1 cross mice. Mice were sacrificed at 24 weeks of age after 12 weeks on a lipid-rich or normal diet. Heart/lung/aortae frozen blocks were prepared, and 6 µm frozen sections prepared on gelatin-coated slides. Sections were either fixed in acetone for 15 90 seconds (for quantitative immunofluorescence; QIF) or in formaldehyde vapor for 18 hours (for histology). Sections were stored at -20°C until analyzed.

Histology. Sections were stained with trichrome stain or hematoxylin/eosin or oil red O/light green for lipid accumulation. Slides fixed in paraformaldehyde were rehydrated, incubated for 18 minutes in fresh oil red O, 20 rinsed, and then incubated 1-2 minutes in fresh light green SF yellowish. The slides were then dehydrated, mounted, and the quantity and position of lipid deposition was analyzed by image analysis.

Quantitative immunofluorescence (QIF). Sections fixed in acetone were rehydrated in TBS + 3% BSA for 30 minutes. The sections were incubated with 25 primary antibody (anti-apo(a) immunosorbed on plasminogen, from Immunex, 1:1000 dilution; anti-total TGF-beta BDA47, from R&D Systems, 1:200 dilution; MBPIIB10, anti-osteopontin antibody, from NIHDSHB, 1:200 dilution) in TBS + 3% BSA. Sections were washed 3 x 3 minutes in PBS, then incubated with fluorescent-labeled second antibody for 2 hours. After washing 3 30 x 3 minutes and mounting, bound fluorescence was quantitated by image analysis. Two markers could be examined on the same section using fluorescein

and rhodamine as distinct fluorescent labels with different excitation and emission characteristics.

Active TGF-beta was localized and quantitated following incubation of slides with fluorescent-labeled extracellular matrix domain of the TGF-beta type II receptor (R2X), expressed in *E. coli* as a glutathione-S-transferase fusion protein.

**Results.** When confluent cells were injured in the presence of serum, many cells migrated into the wound area within 24 hours. Proliferation was also stimulated under these conditions (7% of cells entered DNA synthesis, compared with 3% in an uninjured, control confluent culture). The addition of TGF-beta-1 (10 ng/ml) or tamoxifen (TMX; 10  $\mu$ M) to rat cells at the time of wounding substantially inhibited migration (approximately 90% less cells crossed the boundary of the wound), consistent with previous data that demonstrated that TGF-beta inhibited SMC migration in Boyden Chamber assays. The inhibition of migration by TMX was reversed (>90%) by a neutralizing antibody to TGF-beta-1 (25  $\mu$ g/ml).

In contrast, TGF-beta and TMX did not significantly inhibit the entry into DNA synthesis that was stimulated upon wounding. This observation is consistent with previous data that showed that TGF-beta and TMX slow SMC proliferation by extending the cell cycle in the G<sub>2</sub> phase, rather than by inhibiting or slowing entry into DNA synthesis.

These data agree with previous work that showed that apo(a) inhibits TGF-beta activation in culture, thereby promoting SMC migration. As described in Example 4, apo(a) stimulated VSMC proliferation. Apo(a) is associated with atherogenesis in man and in apo(a) transgenic mice. When apo(a) accumulates in conjunction with reduced levels of active TGF-beta, both migration and proliferation will increase. TMX, which stimulates formation of active TGF-beta, should ameliorate atherogenesis, regardless of whether migration or proliferation (or both) play key roles in pathogenesis.

In adult rat aorta SMC, LDL accumulation is very low, both in freshly dispersed cell preparations and in primary and secondary cultures. This

phenomenon is due to very low levels of LDL receptors (200-400 receptors/cell), irrespective of whether the cells were exposed to lipoproteins.

In contrast, intimal SMC derived from rats 14 days after balloon injury to the carotid artery have a greater ( $\approx 5$  fold) uptake of LDL, due to increased LDL  
5 receptor numbers (1500-2000 receptors/cell). When intimal cells or neonatal cells (displaying very similar properties) are treated with 10 ng/ml TGF-beta for 48 hours, these cells modulate, apparently irreversibly, to the adult phenotype. This phenotypic modulation is accompanied by a down-regulation of LDL  
10 receptors ( $\approx 800$  receptors/cell), with a reduction of LDL uptake of  $>80\%$ . The presence of TGF-beta may therefore reduce lipid accumulation by SMC.

The data obtained with apo(a) transgenic mice are consistent with this prediction. In these mice, apo(a) is accumulated at high levels at the intimal  
15 surface of the aorta. TGF-beta activation is strongly down-regulated from  $>80\%$  in control aortas to  $<20\%$  in apo(a) aortas. Lipid accumulation occurred at these sites in transgenic mice that were fed a lipid-rich diet and had elevated circulating LDL levels. Thus, reduced TGF-beta activity correlates with increased SMC accumulation of LDL from the circulation. TMX, which is capable of elevating TGF-beta *in vivo*, may inhibit lipid accumulation *in vivo*.

#### EXAMPLE 6

##### 20 Effect of Idoxifene on Cultured Human VSMCs

Cultures of human VSMCs were prepared either by enzyme-dispersal using collagenase and elastase or using the explant technique in which cells  
25 migrate out from pieces of aorta (about 1 mm<sup>3</sup>) and proliferate, essentially as described in Example 3. Both enzyme-dispersed (ED) and explant-derived (EX) cultures were prepared from the aortae of two individuals, and either EX or ED cultures were prepared from eight additional donors. The two types of cultures have distinct morphologies and growth characteristics. The EX cultures proliferated much more rapidly than the ED cultures. After six weeks of culturing both types of cultures whenever confluence was attained, the total yield  
30 of cells was approximately 4 fold higher per gram wet weight of aorta in the EX cultures than the ED cultures. Consistent with this observation, the ED cultures

had a longer population doubling time in DMEM + 20% FCS ( $68 \pm 2$  hours;  $n=6$ ) than the EX cultures ( $35 \pm 2$  hours;  $n=6$ ),  $p<0.001$ .

Idoxifene (IDX) is an analog of TMX which has been reported to have enhanced anti-tumor activity (Chandler et al., Cancer Res., **51**, 5851 (1991); McCague et al., Organic Preparation & Proc. Int., **26**, 343 (1994)). The reduced side-effects of IDX compared with TMX and other TMX-related analogs have prompted the selection of IDX for comparison with TMX. IDX at 5  $\mu$ M inhibited increase in cell number by 30% and 28% (two EX cultures tested) compared to control, while cell growth in the presence of 5  $\mu$ M IDX and the neutralizing antibody to TGF- $\beta$  (25  $\mu$ g/ml) was  $95 \pm 6\%$  and  $92 \pm 0\%$  of control. In summary, both TMX and IDX inhibited cell growth of EX-derived, but not ED-derived, hVSMCs to a similar extent ( $ED_{50}=5, 10$  and  $100$ nM;  $n=3$  experiments) and this effect was reversible with the neutralizing antibody to TGF- $\beta$ .

Despite the increasing use of animal models for vascular diseases, such as transgenic mice and balloon-induced injury models, cell culture models of human VSMCs remain important tools because of species-to-species variation. One problem associated with human cell culture models is the potential for variability in properties between individuals due to gender and age, as well as genetic and environmental differences. In this study, it was demonstrated that properties of VSMC cultures derived from ten different donors were very similar. The rate of proliferation, degree of differentiation indicated by expression of the contractile proteins SM- $\alpha$ -actin and SM-MHC and response to growth factors of the smooth muscle cells were not influenced by the age or sex or genetic differences between the individuals.

By contrast, the method of establishing the VSMC culture had marked effects on the properties of the cells. VSMCs derived by the explant technique had a spindle shaped morphology, proliferated rapidly (doubling time of about 35 hours) and lost expression of the contractile protein SM- $\alpha$ -actin and SM-MHC in culture. VSMCs derived from the same individual by the enzyme-dispersal technique were larger, with stellate morphology, proliferated more

slowly (doubling time of about 68 hours) and retained high levels of expression of the contractile proteins SM- $\alpha$ -actin and SM-MHC throughout many (>20) passages in culture. It is therefore important when comparing cell culture studies of human VSMCs to take into account the method used to establish the cultures.

5       The mechanisms which underlie the differences between the two types of human VSMC culture were investigated. All of the differences investigated the potential role of TGF- $\beta$  result from production and activation of TGF- $\beta$  by the ED, but not EX cultures. Addition of a neutralizing antiserum to TGF- $\beta$  to ED cultures altered the properties of the cells so that they resembled EX cells.

10       Conversely, addition of active TGF- $\beta$  to EX cells resulted in properties resembling ED cells. Furthermore, agents previously shown to inhibit rat VSMC proliferation by increasing TGF- $\beta$  activity, such as TMX (Grainger et al., Biochem. J., 294, 109 (1993)) and heparin (Grainger et al., Cardiovas. Res., 27, 2238 (1993)), inhibited the proliferation of EX but not ED cells.

15       A number of recent studies have demonstrated that reduced TGF- $\beta$  activity is correlated with the development of atherosclerosis both in transgenic mouse models (Grainger et al., Nature, 370, 450 (1994)) and in man (Grainger et al., J. Cell. Biochem., 18A, 267 (1994)). The mechanisms which control TGF- $\beta$  production in the ED and EX human VSMC cultures may therefore provide  
20       important clues as to the regulation of TGF- $\beta$  activity *in vivo*. One possibility is that the VSMCs in the ED and EX cultures come from sub-populations of the VSMCs in the vessel wall which differ in their ability to produce TGF- $\beta$ . Evidence is accumulating for heterogeneity of VSMCs both in culture and *in vivo* and it will be informative to determine whether equivalent sub-populations  
25       exist *in vivo* by identifying a number of the genes which are differentially expressed between the two types of culture.

      If a reduction of TGF- $\beta$  activity plays a role in atherogenesis, then agents which elevate TGF- $\beta$  activity, such as TMX, would be expected to reduce the incidence of myocardial infarction. The results described above indicate that  
30       TMX stimulates TGF- $\beta$  production by human VSMC at 10-100 fold lower concentrations than for rat VSMCs. Since TMX was shown to dramatically

reduce the incidence of fatal myocardial infarction in a recent study of 1500 women (McDonald et al., Brit. Med. J., 303, 435 (1994)), it is possible that an increase in active TGF- $\beta$ , operating in an autocrine inhibitory loop, was responsible for these effects.

5

#### EXAMPLE 7

##### Tamoxifen elevates TGF- $\beta$ and suppresses diet-induced formation of lipid lesions in mouse aortae

##### Treatment of Mice with TMX and Preparation of Aortic Sections

Adult (8-12 weeks old) male C57B16 mice in groups were weighed then fed *ad libitum* either normal mouse chow (ICN/Flow), or a high fat diet containing 1.25% cholesterol, 7.5% saturated fat as cocoa butter, 7.5% casein and 0.5% sodium cholate, or high fat diet containing 15  $\mu$ g TMX per gram, or high fat diet containing 1  $\mu$ g TMX per gram. Water was freely available throughout. After three months on the respective diets, each mouse was re-weighed before sacrifice. The heart and attached aorta were embedded in Cryo-M-bed (Bright Instrument Co., Huntington, U.K.) and snap frozen in liquid nitrogen, then 4  $\mu$ m frozen sections were prepared as described previously (Paigen et al., Proc. Nat'l. Acad. Sci., 84, 3763 (1987); Paigen et al., Cancer Res., 45, 3850 (1985)). Platelet-poor plasma was prepared by adding blood taken at the time of death to one tenth volume of 3.5% w/v trisodium citrate on ice. After 15 minutes, the samples were spun (5,000 x g, 15 minutes) and the plasma supernatant retained. In the experiment with 4 groups of 15 mice, the plasma from 9 mice from each group was pooled for analysis of the lipid profile of each group. Separate aliquots from the remaining 6 mice in each group were stored at -20°C until assayed.

25

Measurement of TGF- $\beta$  in Plasma and Aortic Wall Sections The (a+1)TGF- $\beta$  in serum or platelet-poor plasma was measured by ELISA as described above in Example 4. Active TGF- $\beta$  was measured by ELISA using truncated extracellular domain of the type II TGF- $\beta$  receptor (R2X). Active and (a+1)TGF- $\beta$  were measured in 4  $\mu$ m frozen aortic sections by quantitative immunofluorescence as described above in Example 4. Active TGF- $\beta$  was

30

measured using fluorescein-labeled R2X, (a+1)TGF- $\beta$  was measured using BDA19 antiserum (R & D Systems).

Analysis of Lipid Lesion Formation by Oil Red O Staining For each mouse, 5 sections separated by 80  $\mu$ m were fixed in 10% buffered formalin, stained with oil red O and counter stained with light green as described by Paigen et al., *supra*. The first and most proximal section to the heart was taken 80  $\mu$ m distal to the point where the aorta became rounded. The area of oil red O staining in each section was determined with a calibrated eyepiece, excluding lipid droplets less than 50  $\mu$ m<sup>2</sup>, and the mean lesion area per section per mouse was calculated for each mouse and each group of mice. Regions of focal lipid staining >500  $\mu$ m<sup>2</sup> were defined as lipid lesions, and the number of such lesions per section per mouse was determined.

Lipoprotein Profile Analysis One ml of pooled, platelet-poor plasma from each group of mice was diluted to 4 ml with buffer A (0.15 M NaCl, 0.01% (w/v) sodium EDTA and 0.02% (w/v) sodium azide at pH 7.2) and ultracentrifuged at d=1.215 g/ml for 48 hours at 4°C. 0.5 ml of the 2 ml lipoprotein fraction (d<1.215 g/ml) was gel filtered through a sepharose 6B column by FPLC at room temperature. The column was eluted with buffer A at 0.4 ml/minute and fractions of 0.2 ml were collected and analyzed for cholesterol. Cholesterol was measured by the cholesterol oxidase method (Sigma Diagnostics) by adding 5  $\mu$ l from each column fraction to 200  $\mu$ l assay reagent in an ELISA plate (Maxisorp plates; Gibco). The assay plate was incubated at 37°C for 15 minutes and absorbance read at 492 nm. Serum for calibration containing 200 mg/dL total cholesterol (Sigma Diagnostics) was used to convert absorbance readings to cholesterol concentrations according to the manufacturer's instructions. The positions of elution of the major lipoprotein classes in mouse platelet-poor plasma under the conditions described have been determined previously (Yokode et al., *Science*, 250, 1273 (1990)). Fractions 1-9 contained the very low density lipoprotein (VLDL), fractions 10 to 19 contained LDL and fractions above 20 contained HDL.



Assays for Plasma Triglycerides, Cholesterol and Sex Hormones Total plasma triglycerides was measured by the UV end-point glycerol kinase enzymatic method (Sigma Diagnostics). Total plasma cholesterol was measured by the cholesterol oxidase method (Sigma Diagnostics) performed in ELISA plate wells as described above. 17- $\beta$ -estradiol was measured by a specific sandwich ELISA assay (Cascade Biochemicals) and total testosterone plus dihydrotestosterone by radio-immunoassay (Amersham International). All blood parameters (apart from the lipoprotein profile) were performed on six individual platelet-poor plasma aliquots in each group of mice.

- 10 Measurement of SM- $\alpha$ -actin and Osteopontin in Vessel Wall Sections Four  $\mu$ m frozen sections were prepared from the heart/aorta blocks stained with oil red O for lipid lesions. One section adjacent to each section stained for lipid was stained for smooth muscle  $\alpha$ -actin by quantitative immunofluorescence except that the mouse monoclonal antibody to smooth muscle  $\alpha$ -actin, A-2547 (Sigma
- 15 Chemical Co.), was used as the primary antibody at 1:2000 dilution. Fluorescein-labeled anti-mouse IgG (Sigma Chemical Co.) was used as the second antibody at 1:64 dilution. Osteopontin was measured in the next adjacent frozen section, using the mouse monoclonal antibody MBPIIB 10 (NIH Developmental Studies Hybridoma Bank) labeled with biotin followed by
- 20 fluorescein-labeled streptavidin.

### Results

- To determine the effects of TMX on TGF- $\beta$  in the aortic wall and in circulation, an initial study was performed to establish an effective dose. Adult (8 week old) male C57B16 mice (a strain of mice susceptible to lipid lesion
- 25 formation on a high fat diet and which develop fatty streak lesions which resemble the early stages of atherosclerosis in man) in 3 groups were fed *ad libitum* for 28 days on either a normal mouse chow (low fat diet), or a high fat chow containing 0.5% sodium cholate and 5% cholesterol (high fat diet), or high fat diet containing 15  $\mu$ g/g TMX (high TMX diet). The mice on the high TMX
- 30 diet received an average of  $1.1 \pm 0.3$  mg/kg/day of TMX. Groups of 6 mice each were killed at intervals up to 28 days after starting the high TMX diet. Active

TGF- $\beta$  and active plus acid activatable latent TGF- $\beta$  [(a+1)TGF- $\beta$ ] in serum samples and in the aortic wall were determined as described in Example 8. The (a+1)TGF- $\beta$  increased detectably after 3 days reaching a maximum increase of 2.8-fold in serum and 10-fold in the aortic wall and compared with control groups of mice on the high fat diet. After 7 days, (a+1)TGF- $\beta$  in both the vessel wall and in serum declined slowly, so that by 28 days, it was elevated by 2.4-fold in serum and 5.8-fold in the aortic wall. Active TGF- $\beta$  also increased in response to the high TMX diet and the kinetics of the initial increases in active TGF- $\beta$  were very similar to those for (a+1)TGF- $\beta$ , reaching a maximum at 7 days, with more than 90% of the (a+1)TGF- $\beta$  in serum and in the aortic wall was in the active form at 7 days after starting the high TMX diet. However, between 7 and 28 days, the increase in active TGF- $\beta$  in both serum and in the aortic wall decline more rapidly than the (a+1)TGF- $\beta$  so that after 28 days, active TGF- $\beta$  was only elevated by 1.5-fold in serum and 2.2-fold in the aortic wall. The decrease in the proportion of active TGF- $\beta$  after 7 days appears to be due to the induction of plasminogen activator inhibitor-1.

In a further experiment, adult (8 week old) C57B16 mice in 3 groups of 15 were fed on the diets described above, together with a fourth group of 15 mice fed a high fat diet containing 1  $\mu$ g/g TMX (low TMX diet). The mice on the high TMX diet received an average dose of  $1.1 \pm 0.3$  mg/kg/day of TMX on the low TMX diet received  $0.08 \pm 0.02$  mg/kg/day. The remaining mice were killed after 3 months on the diets and the heart, lungs and aortae were embedded and snap-frozen in liquid nitrogen. Platelet-poor plasma was prepared from a terminal bleed. None of the mice in the 4 groups showed anatomical abnormalities, although the mice fed TMX at the high or low doses gained less weight during the period of the experiment than the mice on either the low fat or high fat diet (Table 2). The concentrations of both active and (a+1)TGF- $\beta$  in plasma and in the aortic wall were significantly increased by the high TMX diet. On the low TMX diet, only the active TGF- $\beta$  in plasma did not show a significant increase (Table 2). The effects of TMX on TGF- $\beta$  after 3 months of the high TMX diet were significantly lower than in mice treated for 28 days.

Table 2 - Effects of High Fat Diet and Tamoxifen on C57B16 Mice

	Low Fat	High Fat	Low TMX	High TMX
TMX (mg/kg/day)	-	-	$0.08 \pm 0.02$	$1.1 \pm 0.3$
5 Weight gain over 3 months (g)	$8 \pm 2$	$9 \pm 1$	$5 \pm 2^{**}$	$2 \pm 1^{***}$
(a+1)TGF- $\beta$				
10 Plasma (ng/ml)	$11 \pm 4$	$12 \pm 3$	$18 \pm 5^{**}$	$22 \pm 6^{***}$
Vessel Wall (arbitrary units)	$22 \pm 4$	$20 \pm 2$	$32 \pm 4^{**}$	$44 \pm 8^{***}$
15 Active TGF- $\beta$				
Plasma (ng/ml)	$8 \pm 3$	$8 \pm 2$	$10 \pm 3$	$12 \pm 3^{***}$
20 Vessel Wall (arbitrary units)	$20 \pm 3$	$18 \pm 4$	$28 \pm 3^{**}$	$33 \pm 5^{***}$
Lesions per mouse <sup>a</sup>	$0.7 \pm 0.1$	$3.6 \pm 1.0^*$	$2.6 \pm 0.8^{**}$	$1.1 \pm 0.3^{***}$
25 Lesion area/section/ mouse ( $\mu\text{m}^2$ )	$230 \pm 50$	$6860 \pm 1480^*$	$4660 \pm 960^{**}$	$823 \pm 220^{***}$
17 $\beta$ -estradiol (ng/ml)	$0.28 \pm 0.10$	$0.39 \pm 0.14$	$0.40 \pm 0.20$	$0.25 \pm 0.08$
30 Total Testosterone (ng/ml)	$16 \pm 2$	$14 \pm 3$	$13 \pm 5$	$11 \pm 7$
Total Plasma Cholesterol (mg/dl)	$71 \pm 2$	$92 \pm 4^*$	$79 \pm 3^{**}$	$83 \pm 4^{***}$
35 VLDL Cholesterol (mg/dl)	4	30	38	42

	Low Fat	High Fat	Low TMX	High TMX
LDL cholesterol (mg/dl)	8	33	27	27
5 HDL-cholesterol (mg/dl)	58	27	11	14
Total Triglycerides (mg/dl)	142 ± 15	109 ± 5*	111 ± 9	204 ± 36***
10 SM- $\alpha$ -actin (arbitrary units)	146 ± 6	138 ± 8	168 ± 14	204 ± 12***
15 Osteopontin (arbitrary units)	2 ± 1	46 ± 16*	30 ± 11	5 ± 3***

Serial sections from the aortic sinus region were analyzed for lipid lesions using the oil red O staining protocol and sectioning strategy as described by Paigen et al., *supra*. Small regions of luminal lipid staining were detected in mice on the low fat diet, but most of the vessel wall was devoid of lipid deposits in this group. In mice fed the high fat diet, there was a 5-fold increase in the number of lipid lesions in the aortic wall but in the mice fed the TMX diets, there was a dose-dependent decrease in the number of lesions with a 86% decrease of diet-induced lesions on the high TMX diet (Table 2). The aortic wall area stained with oil red O was measured for each group of mice. Mice on the high fat diet had lesion areas (per section per mouse) of  $6860 \pm 1480 \mu\text{m}^2$  (n=15) consistent with previous published results (Emerson et al., *Am. J. Path.*, 142, 1906 (1993); Paigen et al., *Arteriosclerosis*, 10, 316 (1990)). The high TMX diet and low TMX diets reduced the lesion areas by 88% (n=15;  $p < 0.001$ ) and 32% (n=15;  $p < 0.01$ ) respectively (Table 2). TMX therefore causes a dose-dependent inhibition of diet-induced lipid lesions in C57B16 mice.

High or low TMX diets significantly lowered total plasma cholesterol by approximately 10% compared with mice on the high fat diet. Analysis of the

lipoprotein profiles showed that for the mice on the low fat diet, most of the cholesterol was in the HDL fraction. After 3 months on the high fat diet, however, there was a marked increase in very low density lipoprotein (VLDL) cholesterol of approximately 7-fold (Table 2) and LDL cholesterol (4-fold) whereas the amount of cholesterol in the HDL fraction was reduced by approximately 50% (Table 2). The high and low TMX diets had only small effects on the amount of cholesterol in VLDL or LDL, but further reduced the HDL cholesterol by approximately 50% (Table 2), accounting for most of the overall reduction in cholesterol. In contrast to the decrease in total plasma cholesterol concentration caused by the high TMX diet, there was an increase in plasma concentration of triglyceride (Table 2).

The high or low TMX diets did not affect the very low plasma concentrations of  $17\beta$ -estradiol in the male mice (Table 2). The mean total testosterone concentration (assayed as testosterone plus dihydrotestosterone) was not significantly altered by the TMX diets, although the range of testosterone concentrations was larger than in the mice on the high fat diet, suggesting that TMX may affect testosterone levels in individual mice. However, it is unlikely that changes in the levels of the primary sex hormones in response to TMX are responsible for the inhibition of lipid lesion formation. Medial smooth muscle cells in transgenic apo(a) mice which expressed osteopontin, a marker of de-differentiated smooth muscle cells, are the site of focal apo(a) accumulation and very low TGF- $\beta$  activity. The accumulation of osteopontin occurred in mice on a low fat or high fat diets and was therefore independent of the accumulation of lipid at the sites of low TGF- $\beta$  activity. In the C57B16 mice fed the high fat diet, sections adjacent to the lipid lesions identified by oil red O staining showed regions of high osteopontin accumulation, whereas there was almost no osteopontin accumulation in the aortic sections from mice on the high TMX diet. The type(s) of cells in the aortic wall (e.g., VSMCs, macrophages, etc.) from which the osteopontin was derived, were not identified. Similar experiments in which the accumulation of smooth muscle  $\alpha$ -actin was assayed showed an inverse pattern to that for osteopontin. There were regions of low SM- $\alpha$  actin

expression in adjacent sections to lipid lesions, whereas the amount of SM- $\alpha$  actin was increased in the sections from mice on the high TMX diet. Similar results to those described above for C57B16 mice have been observed in the transgenic apo(a) mouse when these mice were fed a high fat diet. That is, both  
5 the lesion areas and number of lesions for both strains of mice were reduced by approximately 90%.

This example demonstrates that TMX strongly inhibits the formation of lipid lesions induced by a high fat diet in a susceptible strain of mice.

The data show that a major effect of TMX in the C57B16 mice is to elevate  
10 TGF- $\beta$  in aortic wall and in circulation. This is consistent with previous evidence that TMX increases the production of TGF- $\beta$  by VSMCs and other types of cells *in vitro* and in breast tumor cells *in vivo*. The suppression of osteopontin accumulation and the increase in SM- $\alpha$  actin in mice treated with TMX is consistent with previous observations on the apo(a) transgenic mouse  
15 (Example 4). These mice showed large accumulations of osteopontin at sites where focal accumulations of high concentrations of apo(a) result in decreased TGF- $\beta$  activity in the vessel wall. The activation of the smooth muscle cell was also marked by a decrease in local SM- $\alpha$  actin concentration and occurred in the mice on a low fat diet in the absence of lipid accumulation. On a high fat diet,  
20 lipid accumulation occurred at the sites of apo(a) accumulation and lesions formed in two stages: activation of the VSMCs as a result of low TGF- $\beta$  activity and subsequently uptake of lipid by the activated cells when the mice are subjected to a high fat diet. Thus, the cardiovascular protective effect of TMX in mice may be due to elevation of TGF- $\beta$  in the artery wall which prevents VSMC  
25 activation and consequently inhibits lipid accumulation on a high fat diet. TMX causes an overall 2-fold increase in active TGF- $\beta$  in the aortic wall in C57B16 mice and a similar increase in apo(a) transgenic mice would restore the overall TGF- $\beta$  concentration to that observed in normal littermate mice lacking the apo(a) gene. This hypothesis therefore predicts that TMX would prevent lipid  
30 lesion formation in apo(a) mice on a high fat diet. It is of interest that the cardiovascular protective effects of TMX against diet-induced lipid lesions in

mice reported here were obtained at doses similar to those used in breast cancer therapy.

### EXAMPLE 8

#### 5     Determination of Active and Acid Activatable TGF- $\beta$ in Human Sera, Platelets and Plasma by Enzyme-Linked Immunosorbent Assays

Antibodies The antibodies to TGF- $\beta$  used for the ELISAs were BDA19 (a chicken polyclonal IgY antibody which neutralizes TGF- $\beta$  activity) and BDA47 (an affinity purified rabbit polyclonal IgG antibody), both obtained from R&D Systems (Oxford, U.K.). Goat anti-rabbit IgG coupled to horseradish peroxidase was obtained from Sigma Chemical Co. (Poole, U.K.). TGF- $\beta$  standards were obtained from Peninsula (St. Helens, U.K.; purified porcine TGF- $\beta$ 1) and Amersham International (Amersham, U.K.; recombinant human TGF- $\beta$ 1). To refer the ELISA data obtained with these TGF- $\beta$ 1s to the interim international standard, bovine TGF- $\beta$ 1 (89/516) was obtained from the National Institute of Biological Standards and Control (Potters Bar, U.K.). TGF- $\beta$ 2 and TGF- $\beta$ 3 isoforms were obtained from R&D Systems). The TGF- $\beta$  standards were dissolved in 25 mM Tris/HCl pH 7.4 containing 50  $\mu$ g/ml fatty acid free bovine serum albumin (FAF-BSA) to give 5  $\mu$ g/ml stock solutions. The concentration of the standard TGF- $\beta$  solutions was checked against the bioassay of DNA synthesis in MvLu epithelial cells (see below). Both TGF- $\beta$  standards gave an ED<sub>50</sub> for inhibition of DNA synthesis in the MvLu bioassay of between 2-3pM which agrees well with the previously reported value of 2pmol/L (Danielpur et al., J. Cell Physiol., 138, 79 (1989)).

25     Growth Factors Platelet-derived growth factor (PDGF) AA and BB homodimers and epidermal growth factor (Bachem Inc., Saffron Walden, U.K.) were dissolved in 25 mmol/L Tris/HCl, pH 7.4 containing 1% FAF-BSA to give 0.3  $\mu$ mol/L stock solutions. Basic fibroblast growth factor (0.56  $\mu$ mol/L) interleukin 1  $\beta$  (0.59  $\mu$ mol/L), transforming growth factor  $\alpha$  (1.81  $\mu$ mol/L), 30     interferon  $\gamma$  (0.59  $\mu$ mol/L) and insulin-like growth factor I (0.59  $\mu$ mol/L; all from Bachem Inc.) were dissolved in sterile MilliQ water to give stock solutions of the concentrations indicated. Angiotensin II and endothelin I (Sigma

Chemical Co.) were dissolved in sterile MilliQ water to give 10  $\mu\text{mol/L}$  stock solutions.

- Recombinant Expression of the TGF- $\beta$  Type II Receptor The extracellular domain of the TGF- $\beta$  type II receptor was amplified from the vector H2 3FF (Lin et al., *Cell*, 68, 775 (1992)) using the polymerase chain reaction (PCR). The vector DNA was linearized with Not I, precipitated and resuspended at 10 ng/ $\mu\text{L}$ . Amplification was carried out in a 50  $\mu\text{L}$  reaction containing 2.5  $\mu\text{L}$  DNA, 5  $\mu\text{L}$  10x TAQ buffer (LKB Pharmacia; Upsalla, Sweden), 250 ng of each oligonucleotide primer (GAATTC<sup>3</sup>CCATGGGTCGGGGGCTGCTC (SEQ ID NO:1) and GAATTCGTCAGGATTGCTGGTGT (SEQ ID NO:2); Wellcome Protein and Nucleic Acid Chemistry Facility, University of Cambridge), 1 U TAQ polymerase and a mixture of dATP, dTTP, dCTP and dGTP to give a final concentration of 200  $\mu\text{M}$  for each nucleotide. The sample was overlaid with 50  $\mu\text{L}$  paraffin oil. The reaction was carried out using a thermal cycler (PREM; Cambridge, U.K.) for 30 cycles (denaturing at 94°C for 1 minute, annealing at 55°C for 2 minutes, elongation at 72°C for 2 minutes). The 450 bp fragment produced was purified by electrophoresis in low gel temperature agarose, digested with EcoRI and cloned into the glutathione-S-transferase fusion vector pGEX 2T (LKB Pharmacia). Vectors carrying inserts in the required orientation were identified by plasmid mapping. The sequence of the insert was checked by subcloning the 450 bp EcoRI fragment from the chosen clone (pGT1C) into Bluescript KS+ followed by double strand sequencing. The sequence showed a single base change (C to A at position +13 from the initiation codon) compared to the published sequence (Lin et al., *supra*.) which introduces a leu to met mutation in the protein.

- Protein Purification An overnight culture of *E. coli* TG1 containing pGT1C was diluted 1:100 into fresh 2YT medium (500 mL) containing 270  $\mu\text{mol/L}$  ampicillin and grown to an  $\text{OD}_{600}$  of 0.5. Production of the fusion protein was induced by addition of 1 mM isopropylthiogalactoside and the cells were harvested 5 hours later by centrifugation. The bacteria were resuspended in 50 mL phosphate buffered saline (PBS; 150 mmol/L NaCl, 2 mmol/L  $\text{Na}_2\text{HPO}_4$ ).



4 mmol/L  $\text{Na}_2\text{HPO}_4$ , pH 7.3) containing 1% Triton X-100 and 1 mmol/L PMSF and lysed by sonication for 5 minutes. The lysate was centrifuged ( $10,000 \times g$ ; 5 minutes) and the fusion protein was purified from the supernatant by the one step purification method of Smith and Johnson (*Gene*, 67, 31 (1988)). FPLC of the  
 5 purified glutathione-binding proteins on a Superdex 200 HR column in 20 mM ammonium bicarbonate, pH 8.0, demonstrated that >95% of the protein present was the desired 43 kDa TGF- $\beta$  receptor fusion protein.

ELISA to Measure Total TGF- $\beta$  Maxisorp 96 well ELISA plates (Gibco; Uxbridge, U.K.) were coated with the capture antibody by incubating with 50  $\mu\text{L}$   
 10 BDA19 anti-TGF- $\beta$  chicken IgY (40  $\mu\text{g}/\text{mL}$ ) diluted in Tris-buffered saline (TBS; 137 mmol/L NaCl, 50 mmol/L Tris/HCl, pH 7.4) and shaking the plates until dry by evaporation at room temperature (approximately 12 hours). The plates were washed 3 x 3 minutes with PBS, blocked with 350  $\mu\text{L}$  3% FAF-BSA in TBS for 1 hour, washed 3 x 3 minutes with TBS and incubated for 2 hours  
 15 with 100  $\mu\text{L}$  of test samples or dilutions of a TGF- $\beta$  stock solution for calibration. The purified porcine TGF- $\beta$  stock solution diluted in TBS to concentrations between 0.4 pmol/L and 4000 pmol/L was used for calibration unless otherwise indicated.

The plates were washed (3 x 3 minutes) with TBS + 3% FAF-BSA + 0.1%  
 20 Triton X-100 (wash buffer) and incubated with 20  $\mu\text{L}$  detection antibody (BDA47; anti-TGF- $\beta$  (rabbit IgG)) at 1  $\mu\text{g}/\text{mL}$  in wash buffer for 1 hour. The plates were rinsed with wash buffer (3 x 3 minutes) and incubated with an antibody against rabbit IgG conjugated to horseradish peroxidase (Sigma A-6154) at 1:2500 dilution in wash buffer for 1 hour. After washing (3 x 3 minutes  
 25 with wash buffer), the plates were incubated for 15 minutes with the chromogenic substrate orthophenylenediamine (Sigma) according to the manufacturer's instructions. The reaction was stopped by addition of an equal volume of 3M HCl and the absorbances read on an ELISA plate reader (Titertek Multiscan; Flow Laboratories, High Wycombe, U.K.) within 15 minutes of  
 30 stopping the reaction. Absorbances were converted into quantities of TGF- $\beta$  protein using the calibration curve from the TGF- $\beta$  standard.

ELISA to Measure Active TGF- $\beta$  This ELISA was performed as for the ELISA to assay total TGF- $\beta$  except: (i) the ELISA plates were coated with the purified TGF- $\beta$  receptor fusion protein using 20  $\mu$ L of a 50  $\mu$ g protein per mL of solution in TBS and (ii) the detection reagent (BDA47) was used at 5  $\mu$ g/mL.

- 5 Mink Lung Epithelial DNA Synthesis Bioassay Mink lung epithelial cells (MvLu; American Type Culture Collection; passage 49-60) were subcultured at 1:5 dilution in DMEM + 10% FCS. After 24 hours, the medium was replaced with DMEM + 10% FCS containing the sample (< 1% v/v) or standards in the presence and absence of neutralizing antiserum to TGF- $\beta$  (BDA19) at 10  $\mu$ g/mL.
- 10 DNA synthesis during a 1 hour pulse of 6-[ $^3$ H]-thymidine (5  $\mu$ Ci/mL; Amersham International) was determined 23 hours after addition of test medium. TGF- $\beta$  activity was calculated as the proportion of the inhibition of DNA synthesis which was reversed in the presence of neutralizing antibody, using a standard curve to convert the inhibition values into quantities of active TGF- $\beta$ . Purified
- 15 porcine TGF- $\beta$  diluted in TBS was used as the standard unless otherwise indicated.

- Preparation of Conditioned Culture Media. Human Platelets. Platelet-Poor Plasma and Serum Medium (DMEM + 20% FCS) was conditioned for 24 hours on cultures of adult human aortic VSMCs obtained by enzymatic dispersion of
- 20 aortic media as described above.

- Twenty mL of peripheral venous blood was collected from 12 healthy male volunteers (aged 23-54); 10 mL were aliquoted immediately into tubes containing 1.1 mL of sterile 3.8% (w/v) trisodium citrate in MilliQ water at room temperature. The samples were centrifuged (250 x g; 15 minutes) to remove red
- 25 blood cells. Apyrase (Sigma) was added to the platelet-rich plasma to a final concentration of 100 mg/L to prevent platelet degranulation; PMSF (1 mmol/L) and aprotinin (1 mg/L) were added to prevent proteolytic activation or degradation of TGF- $\beta$ . These samples were centrifuged (700 x g; 15 minutes) and the supernatant platelet-poor plasma was separated from the platelet pellet.
- 30 The platelet-poor plasma was kept at room temperature until assayed by ELISAs within 2 hours of preparation or was stored in 0.5 mL aliquots at -80°C. The

platelet pellet was resuspended in 10 mL (i.e., the original volume of blood) of a buffered saline solution (145 mmol/L NaCl, 5 mmol/L KCl, 10 mmol/L glucose, 10 mmol/L MgSO<sub>4</sub>, 0.5 mmol/L EGTA, 1 mmol/L PMSF, 1 mg/L aprotinin, 10 mmol/L HEPES, pH 7.4) and recentrifuged as before. The washed platelet pellet was resuspended in 10 mL of buffered saline solution and the platelet concentration was determined by hemocytometer. Platelets were lysed by ultrasonication until <10% of unlysed platelets were detected by hemocytometer. Human platelet suspensions were also obtained from the Blood Transfusion Service, Cambridge, U.K. The platelets were collected by centrifugation (3,000 x g; 3 minutes) and approximately 0.1 g of platelets were resuspended in 0.5 mL MilliQ water and lysed by three cycles of freeze-thawing. The membrane fragments were removed by centrifugation (14,000 x g; 10 minutes) and the supernatant was mixed with an equal volume of 2 x TBS.

The remaining 10 mL of freshly drawn blood samples were dispensed immediately into polypropylene tubes and allowed to clot at room temperature for 2 hours. The clotted samples were centrifuged (1,000 x g; 4 minutes), the serum was removed and either stored on ice until assayed within 2 hours or stored at -80°C until assayed. The clot was washed three times by centrifugation (1000 x g; 4 minutes) in 5 mL of 150 mM phosphate buffer, pH 7.0, and the third wash was retained for TGF- $\beta$  assays. The washed clot was dissolved in 5 mL of 150 mM phosphate buffer, pH 2.0, for 30 minutes, then neutralized by addition of 5 mL of 150 mM phosphate buffer, pH 12.0. The samples were assayed for TGF- $\beta$  immediately or stored in 1 mL aliquots at -80°C.

All blood-derived samples, stored at -80°C, were not thawed until assayed. The initial freeze-thaw cycle resulted in less than 10% loss of total or active TGF- $\beta$  activity in the ELISAs. However, three additional freeze-thaw cycles of samples containing TGF- $\beta$  in active or latent form was sufficient to cause loss of approximately 90% activity.

**Bioassays of PDGF** PDGF was bioassayed by its mitogenic activity on human VSMCs derived by explant as described previously (Kocan et al., Methods in Cell Biology, eds. Harris, C.C., Trump, B.F., and Stenes, G.D., Academic Press

(1980)). VSMCs were made quiescent by incubation in serum-free DMEM for 48 hours. Samples of serum or platelet-poor plasma were added at a final concentration in DMEM of 5% or 20%, respectively. DNA synthesis was assayed by [<sup>3</sup>H]-thymidine (Amersham International; 5 µCi/mL) incorporation between 12 hours and 36 hours after addition of the test samples to the cells. The proportion of DNA synthesis due to PDGF was estimated by the addition of polyclonal antibody (50 mg/L) which neutralizes all forms of PDGF to replicate cell samples.

- 10 **Results** An ELISA was set up to detect total ( $\alpha + \beta$ ) TGF- $\beta$  using the polyclonal chicken IgY antibody BDA19 as the capture reagent. The assay detected purified porcine TGF- $\beta$  in TBS in the range of 4 pmol/L to 2000 pmol/L with half-maximal change in absorbance ( $\Delta A_{50\%}$ ) of  $280 \pm 80$  pmol/L (n=7). Using recombinant human TGF- $\beta$ 1 in TBS, the assay detected TGF- $\beta$  in the range
- 15 8 pmol/L to 2000 pmol/L with a  $\Delta A_{50\%}$  of  $320 \pm 120$  pmol/L (n=3). Direct comparison of the TGF- $\beta$ 1 (R&D Systems) was made with the interim international bovine TGF- $\beta$  (89/516). An ampoule of 89/516 containing 1500 units (approximately 80 ng protein; 32 pmol) was dissolved in sterile water to 800 µl and serially diluted in TBS and similar dilutions of the R&D Systems
- 20 TGF- $\beta$ 1 made. Comparison of the calibration curves showed that a nominal 1.0 pmol at R&D TGF- $\beta$ 1 had an activity of  $130 \pm 8$  units. To test the specificity of the capture antibody in the total TGF- $\beta$  assay, it was replaced with nonimmune chicken IgY (R&D Systems). The change in absorbance in the presence of 4000 pmol/L of purified porcine TGF- $\beta$ 1 was less than 5%,
- 25 indicating that TGF- $\beta$  binding under the assay conditions was specific to the capture agent.

- To test whether the ELISA detected acid activatable, latent forms of TGF- $\beta$ , a sample of human platelets from the blood bank was lysed and assayed before and after activation of the TGF- $\beta$  (Wakefield et al., J. Biol. Chem. 263: 7646 (1988); Assoian et al., J. Cell Biol. 102: 1031 (1986)). The latent TGF- $\beta$
- 30 was converted to active TGF- $\beta$  by addition of 5% vol/vol 150 mmol/L sodium

phosphate buffer at pH 2.0 for 5 minutes, then neutralized by addition of 5% vol/vol 150 mmol/L sodium phosphate buffer at pH 12.0 (Barnard et al., Biochim. Biophys. Acta, 1032, 79 (1990)). Control samples were treated with 10% vol/vol 150 mmol/L sodium phosphate buffer at pH 7.0. The MyLu cell

- 5 bioassay of the untreated and acid-treated platelet lysate showed that the amount of active TGF- $\beta$  was increased 5.1-fold after acid activation of the latent TGF- $\beta$ , indicating that approximately 80% of the TGF- $\beta$  present in the unactivated sample was in the acid activatable, latent form. When assayed by the total TGF- $\beta$  ELISA, the control aliquot contained  $680 \pm 80$  pmol/L TGF- $\beta$  (n=3) by
- 10 ELISA and the acid-activated aliquot contained  $600 \pm 120$  pmol/L TGF- $\beta$  (n=3). These results show that the total TGF- $\beta$  ELISA does not distinguish between active and acid activatable TGF- $\beta$  from human platelets.

- The precise conditions for activation of the small and large complexes of latent TGF- $\beta$  have not been characterized and there is some evidence for the
- 15 existence of two pools of latent TGF- $\beta$  which differ in the conditions required for activation. Therefore, TGF- $\beta$  is defined as that pool of latent TGF- $\beta$  which is acid-activatable by the treatment described above (i.e., exposure to pH 2.0 for 5 minutes before neutralization to pH 7.0 without overshoot). Longer exposure to pH 2.0 did not significantly affect the concentration of activated TGF- $\beta$  and it
- 20 remains to be determined which form(s) of latent TGF- $\beta$  are activated under the defined conditions.

- A second ELISA was established to measure active TGF- $\beta$  in the presence of latent TGF- $\beta$  using a truncated TGF- $\beta$  type II receptor protein fused to glutathione-S-transferase as the capture reagent. This assay detected purified
- 25 porcine TGF- $\beta$ 1 in TBS in the range of 20 pmol/L to 4000 pmol/L with a  $\Delta A_{30\%}$  of  $680 \pm 160$  pmol/L (n=4) and recombinant human TGF- $\beta$ 1 in TBS in the range of 40 pmol/L to 4000 pmol/L with a  $\Delta A_{30\%}$  of  $720 \pm 120$  pmol/L (n=3). To test the specificity of the truncated receptor fusion protein as the capture agent, it was replaced with glutathione-S-transferase. The change in absorbance in the present
- 30 of 4000 pmol/L of purified porcine TGF- $\beta$ 1 was less than 5%, indicating that TGF- $\beta$  binding was specific to the capture agent under the assay conditions.

To confirm that the active TGF- $\beta$  ELISA did not detect acid activatable, latent-TGF- $\beta$ , samples of human platelet TGF- $\beta$  before and after acid activation were assayed. The active TGF- $\beta$  ELISA gave  $160 \pm 40$  pmol/L ( $n=3$ ) in the unactivated sample and  $640 \pm 80$  pmol/L ( $n=3$ ) TGF- $\beta$  in the acid-activated sample, consistent with the data obtained from the ( $\alpha$ +I) TGF- $\beta$  ELISA and the MvLu cell bioassay described above. The ability of the ELISA to discriminate between active and latent TGF- $\beta$  was further defined in studies on TGF- $\beta$  in fresh human platelets (see below).

To test the reproducibility of both ELISAs, 24 aliquots of a sample of lysed human platelets from the blood bank was assayed simultaneously by both assays. The value for active TGF- $\beta$  was 200 pmol/L with a coefficient of variation of 7.4% and the corresponding value for ( $\alpha$ +I) TGF- $\beta$  was 640 pmol/L with a coefficient of variation of 6.8%. Further aliquots of the same platelet lysate were also analyzed blind by four independent operators using both ELISAs on eight separate occasions. The inter-assay coefficient of variation was 13.2% for the active TGF- $\beta$  assay and 12.2% for the ( $\alpha$ +I) TGF- $\beta$  assay.

The relative sensitivity of each ELISA to the three isoforms of TGF- $\beta$  was determined. Recombinant human TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3 (400 pmol/L) in TBS were assayed using each ELISA, expressing the absorbance for TGF- $\beta$ 2 and TGF- $\beta$ 3 as a percentage of the absorbance for TGF- $\beta$ 1. Both ELISAs detect TGF- $\beta$ 1 and TGF- $\beta$ 3 with similar sensitivity, but TGF- $\beta$ 2 was detected with approximately 10-fold less sensitivity than the other isoforms in the ( $\alpha$ +I) TGF- $\beta$  ELISA and 100-fold less sensitivity in the active TGF- $\beta$  ELISA. The relative sensitivities for the isoforms in the active TGF- $\beta$  ELISA are qualitatively consistent with the relative TGF- $\beta$  isoform affinities of the type II TGF- $\beta$  receptor (Massagué, *Ann. Rev. Cell Biol.*, 6, 597 (1990)). The slightly greater relative sensitivity of the active TGF- $\beta$  ELISA to TGF- $\beta$ 3 than the ( $\alpha$ +I) TGF- $\beta$  ELISA would result in an overestimate of the proportion of active TGF- $\beta$  in a sample which was composed mostly of TGF- $\beta$ 3 if the assays were calibrated using a TGF- $\beta$ 1 standard. The proportion of active TGF- $\beta$  in samples containing only the TGF- $\beta$ 2 isoform cannot be determined accurately by these ELISAs at

concentrations below 4000 pmol/L. The concentration of TGF- $\beta$ 2 in human serum has been reported as <5 pmol/L (Danielpur et al., Annals N.Y. Acad. Sci., 593, 300 (1990)).

The cross-reactivity of both ELISAs to a variety of other peptide growth factors was determined at concentrations which have a maximal biological effect in cell culture. Neither assay gave a change of greater than 5% in absorbance in response to PDGF-AA (3.3 nmol/L), PDGF-BB (3.3 nmol/L), basic fibroblast growth factor (5.6 nmol/L), epidermal growth factor (15.9 nmol/L), insulin-like growth factor I (1.3 nmol/L), angiotensin II (100 nmol/L), endothelin I (100 nmol/L), interleukin 1 $\beta$  (588 pmol/L), transforming growth factor  $\alpha$  (1.8 nmol/L), or interferon  $\gamma$  (588 pmol/L).

There are several reports that TGF- $\beta$  binds to serum components and extracellular matrix components with high affinity. For example, McCaffrey and co-workers demonstrated that TGF- $\beta$  associates non-covalently with the major serum protein,  $\alpha$ 2-macroglobulin (J. Cell Biol., 109, 441 (1986)). However, preparation of the TGF- $\beta$  standard solutions in the presence of 1.4  $\mu$ mol/L human  $\alpha$ 2-macroglobulin or 10% FCS did not affect the  $\Delta A_{50\%}$  by more than 10% compared with the  $\Delta A_{50\%}$  for the standard TGF- $\beta$  solutions diluted in TBS in either ELISA. Therefore, any non-covalent interactions formed between TGF- $\beta$  and  $\alpha$ 2-macroglobulin or with components of FCS do not prevent active TGF- $\beta$  from binding to the type II TGF- $\beta$  receptor in the active TGF- $\beta$  ELISA or to the capture antibody in the ( $\alpha$ +I) TGF- $\beta$  ELISA, nor do they inhibit binding by the detection antibody. It has been noted in a previous report that purified TGF- $\beta$  and  $\alpha$ 2-macroglobulin may not interact in the same way as endogenous serum TGF- $\beta$  and  $\alpha$ 2-macroglobulin (O'Conner-McCorua et al., J. Biol. Chem., 262, 14090 (1987)).

The active TGF- $\beta$  concentration was measured in three samples of medium (DMEM containing 10% FCS) conditioned for 24 hours on human VSMCs which produce active TGF- $\beta$ . The values obtained with the active TGF- $\beta$  ELISA were compared with those obtained using the MvLu cell bioassay (Table 3).

**Table 3**  
Active TGF- $\beta$  concentration in medium  
conditioned on human VSMCs

5 Active TGF- $\beta$  (pM)

Sample	MvLu Assay	Active TGF- $\beta$ ELISA
1	584 $\pm$ 24	552 $\pm$ 32
2	356 $\pm$ 32	400 $\pm$ 24
3	488 $\pm$ 40	484 $\pm$ 16

10

The amount of active TGF- $\beta$  present in three different samples of DMEM + 20% FCS which had been conditioned on human VSMC cultures for 24 hours was determined in quadruplicate using the DNA synthesis bioassay in MvLu epithelial cells and the active TGF- $\beta$  ELISA.

15

The results obtained by the two assays were not statistically different for any of the three samples tested ( $p = 0.88, 0.48$  and  $0.99$ , using students unpaired t-test). Thus, the ELISA gives values for active TGF- $\beta$  concentrations in conditioned medium which are closely consistent with the MvLu cell bioassay used previously. Where possible, it is important to demonstrate consistency between the active TGF- $\beta$  ELISA and the bioassay for conditioned media and other biological fluids. For example, it has recently been reported that direct addition of conditioned media to ELISA microwells can lead to inaccurate measurement of TGF- $\beta$  for reasons that are not fully understood (Danielpur, J. Immunol. Methods, 158, 17 (1993)). Protocols which activate and concentrate TGF- $\beta$ s to partially purify the samples and exchange the buffer were recommended (Danielpur, supra).

20

25

30

Another factor which might interfere with the assays is any peroxidases present in serum which bind to the capture reagents. To test for peroxidases, the capture antibody in the ( $\alpha$ -1) TGF- $\beta$  assay was replaced with non-immune chicken IgY, and the truncated receptor fusion protein in the active TGF- $\beta$  assay was replaced with glutathione-S-transferase. The change in absorbance in either



assay was less than 5% in the presence of either DMEM containing 10% FCS or human serum from donors A, E, K, or N in Table 5. These data indicated that any peroxidase activity in FCS or human serum did not significantly affect the assays of ( $\alpha$ +l) or active TGF- $\beta$ s.

5

Table 4

Active and ( $\alpha$  + l) TGF- $\beta$   
concentrations in human sera

Donor	TGF- $\beta$ (pmol/L)			
	Unactivated serum		Acid-activated serum	
	Active	( $\alpha$ + l)	Active	( $\alpha$ + l)
A	<40	240	240	240
B	120	120	120	120
C	200	320	320	320
D	240	240	240	240

10

15

Serum samples from four male donors were assayed in a single experiment for active and total TGF- $\beta$  by the ELISAs before and after acid activation. All samples were assayed in quadruplicate.

The above experiments suggested that the ELISAs could be used to measure TGF- $\beta$  in human serum and the use of the assays for sera was therefore characterized. It was found that the calibration curves for both the active and ( $\alpha$ +l) TGF- $\beta$  assays were not affected when purified porcine TGF- $\beta$  was added to human serum (donor E in Table 5) which contained very little TGF- $\beta$  by either ELISA.

25

**Table 5**

( $\alpha$ +I) and active TGF- $\beta$  concentrations  
in human serum samples

Donor	TGF- $\beta$ (pmol/L)		
	Active	( $\alpha$ +I)	% active
E	<20	<4	-
F	<20	<4	-
A	<20	240	<8
G	20	80	25
H	80	80	100
I	80	80	100
J	80	120	66
K	160	1120	14
C	280	320	88
L	320	320	100
M	360	320	113
N	1400	1400	100

Serum samples from 12 male donors aged between 23 and 54 were assayed immediately after preparation for active and ( $\alpha$ +I) TGF- $\beta$  by the ELISAs described. All samples were assayed in quadruplicate by each ELISA in a single experiment.

- For human sera comparisons of active TGF- $\beta$  concentrations by the ELISA and the MvLu cell bioassay were not possible because human serum inhibited MvLu DNA synthesis by a mechanism independent of TGF- $\beta$ . The presence of 10% (v/v) serum from any of 4 donors (A, H, J, and K in Table 5) inhibited DNA synthesis in MvLu cell cultures by more than 95%. This inhibition was not reversed by the presence of neutralizing antibodies to TGF- $\beta$ , indicating that the human sera contained an inhibitor of DNA synthesis in MvLu cells which masked any effect of TGF- $\beta$ . The MvLu cell bioassay cannot therefore be used

to determine the concentration of active TGF- $\beta$  in unfractionated human serum samples.

Alternative approaches were therefore required to validate the ELISA assays for direct use with human serum. The main requirement was to determine whether human sera contain non-TGF- $\beta$  components which significantly affected the TGF- $\beta$  concentrations estimated by either assay. Overestimated values of TGF- $\beta$  would be obtained if a serum component was bound specifically or nonspecifically by the capture agent in either assay and was also recognized by the detection antibody or by the antibody to rabbit IgG linked to horseradish peroxidase. Alternatively, underestimated values would result if a serum component competed with TGF- $\beta$  for the capture agent in either assay but was not recognized by the detection antibody. In a previous study in which TGF- $\beta$  in unfractionated serum (after transient acidification) was determined by a radio-receptor assay, it was found that components in the serum interfered with the assay (O'Connor-McCourt et al., *J. Biol. Chem.*, **262**, 14090 (1987)). This resulted in a dilution curve which was not parallel to the standard dilution curve and estimates of TGF- $\beta$  were 20 to 40 times lower than those obtained by acid-ethanol extraction of the same samples. Thus, it is possible that serum components which result in either overestimated or underestimated TGF- $\beta$  values in our ELISAs would also interfere with other assays (receptor binding or radio-immunoassays) used to validate serum TGF- $\beta$  concentrations estimated by the ELISAs. Therefore, a more rigorous test for interfering components in serum was required. This was achieved by determining whether the concentrations of active and ( $\alpha$ +I) TGF- $\beta$  concentrations in sera were internally consistent before and after activation of latent TGF- $\beta$  by acid treatment. Only under very implausible circumstances would consistent accounting of active and ( $\alpha$ +I) TGF- $\beta$  be obtained in the presence of serum components which interfered with either or both assays.

ELISAs of ( $\alpha$ +I) and active TGF- $\beta$  concentrations were performed on the sera from 4 male donors before and after the sera were acidified to pH 2.0 and neutralized to pH 7.0 as described for the lysed human platelet samples. For

each of the sera in Table 4, there was no difference within the accuracy of the assays between the amount of ( $\alpha$ +I) TGF- $\beta$  before and after acid treatment. Furthermore, after acid treatment, the amount of active TGF- $\beta$  was not significantly different from the amount of ( $\alpha$ +I) TGF- $\beta$ . These results imply that it is very unlikely that the sera tested contained components which interfered with either TGF- $\beta$  ELISA since they would cause significant imbalances in the quantitative accounting of the amounts of active and ( $\alpha$ +I) TGF- $\beta$  before and after acid treatment. The use of acid treatment of the sera and reassay of the active and ( $\alpha$ +I) TGF- $\beta$  concentrations therefore provides an important internal control for the TGF- $\beta$  assays when used directly for sera or complex biological fluids.

The sera from 12 male donors (aged 23 to 54) were assayed for active and ( $\alpha$ +I) TGF- $\beta$  by the ELISAs (Table 5). The mean ( $\alpha$ +I) TGF- $\beta$  concentration was 330 pmol/L, but the variation was very large (range less than 4 pmol/L to 1400 pmol/L). Similarly, the mean active TGF- $\beta$  concentration was 230 pmol/L, and the range was from less than 20 pmol/L to 1400 pmol/L. The proportion of the ( $\alpha$ +I) TGF- $\beta$  present which was active ranged from <10% to 100% with a mean of 73% for the samples for which percent activation could be determined. These data for the amount of TGF- $\beta$  in human serum can be compared with several previous reports. A value of  $4.2 \pm 0.7$  pmol/L ( $n=10$ ) active TGF- $\beta$  was obtained using the IL-4 dependent HT-2 cell proliferation assay (Chao et al., Cytokine, 3, 292 (1991)). However, when the serum was treated with acid, an increase of greater than 100-fold in TGF- $\beta$  values was detected by the same proliferation assay. This implies a mean value for activatable (i.e., ( $\alpha$ +I)) TGF- $\beta$  of >420 pmol/L. In an earlier study (O'Connor-McCourt et al., supra) using both a two-step competitive radio-receptor assay and the NRK cell-soft agar growth system, it was reported that acid-ethanol-extraction of serum (FCS, calf and human) gave ( $\alpha$ +I) TGF- $\beta$  concentrations of 200-1000 pmol/L. A value for human serum for TGF- $\beta$ 1 of 1,300 pmol/L and <5 pM for TGF- $\beta$ 2 measured by specific ELISAs has also been reported (Dasch et al., Annals N.Y. Acad. Sci., 593, 303 (1990)). Of these data, only the low active TGF- $\beta$  value of  $4.2 \pm$

0.7 pmol/L (n=10) differs substantially from the range of our ELISA values for human sera (Chao et al., *supra*).

Platelet-poor plasma samples were prepared from the same blood samples used to prepare sera from the 4 donors in Table 4. There was no difference within the accuracy of the assays between the amount of ( $\alpha$ +I) TGF- $\beta$  before or after acid treatment of the plasma samples, and after acid treatment, the amount of active TGF- $\beta$  was not significantly different from the amount of ( $\alpha$ +I) TGF- $\beta$  (Table 6).

Table 6

Active and ( $\alpha$ +I) TGF- $\beta$  concentrations  
in human platelet-poor plasma

Donor	TGF- $\beta$ (pmol/L)			
	Unactivated plasma		Acid-activated plasma	
	Active	( $\alpha$ +I)	Active	( $\alpha$ +I)
A	<40	240	240	240
B	120	120	120	120
C	160	320	320	320
D	200	240	240	280

Platelet-poor plasma were derived from the same blood samples as the sera for Table 4 and were assayed in the same experiment for active and ( $\alpha$ +I) TGF- $\beta$  by ELISA before and after acid activation. All samples were determined in quadruplicate.

These data demonstrate that the plasma did not contain components which interfered with either ELISA, consistent with the finding for the sera derived from the same blood samples.

Comparison of the data in Tables 4 and 6 also shows that ( $\alpha$ +I) TGF- $\beta$  concentrations and the proportions of TGF- $\beta$  which were active were very similar in serum and platelet-poor plasma prepared from the same blood samples. These data implied that either the platelets had degranulated to release their TGF- $\beta$  during the preparation of the platelet-poor plasma so that the

amounts of TGF- $\beta$  were the same in plasma and in serum, or that platelet degranulation during clotting in the preparation of serum did not release active or latent TGF- $\beta$  into the serum. The serum and plasma TGF- $\beta$  concentrations would then be similar because the serum and plasma did not contain a significant  
5 amount of active or latent TGF- $\beta$  from platelets which had degranulated after drawing the blood samples.

To examine whether the active or latent TGF- $\beta$  in the serum and plasma samples was derived from degranulation of platelets after drawing blood, ( $\alpha$ +I) TGF- $\beta$  concentrations in the sera, acid-extracted clots, platelet-poor plasma and  
10 platelets from seven donors were compared (Table 7).

Table 7

( $\alpha$ +I) TGF- $\beta$  concentrations in human serum, plasma, platelets, and acid-treated clots

Donor	( $\alpha$ +I) TGF- $\beta$ (pmol/L)			
	Serum	Platelet-poor plasma	Platelets	Acid-treated clot
E	<40	40	1000	960
N	80	80	880	760
B	120	120	1000	1200
D	280	280	1600	1600
A	320	360	1200	1200
C	440	440	1000	720
M	1200	1400	760	760

Serum, platelet-poor plasma and platelets were prepared from blood from 7 male donors. Clots were removed from the serum samples by centrifugation, washed, dissolved by acidification and neutralized. TGF- $\beta$  was released from platelets by sonication which lysed >90% of the platelets present. ( $\alpha$ +I) TGF- $\beta$  in each sample was assayed by ELISA in quadruplicate. TGF- $\beta$  concentrations for platelets and clots are calculated for the volume of blood from which they were derived.

The ( $\alpha$ +I) TGF- $\beta$  concentrations in serum and plasma derived from the same blood samples were very similar, consistent with the data in Tables 4 and 6. The average concentration of ( $\alpha$ +I) TGF- $\beta$  from the degranulated platelet samples was 1063 pmol/L and the average platelet concentration by hemocytometer in the platelet preparations was  $3.0 \times 10^{11}$ /L, equivalent to an average of 2,100 molecules of TGF- $\beta$  per platelet. This may be compared with a previous estimate of 500 to 2,000 molecules of TGF- $\beta$  per platelet recovered from "platelet secretate" (Wakefield et al., *J Biol. Chem.*, 263, 7646 (1988)). However, the surprising observation was that the ( $\alpha$ +I) TGF- $\beta$  concentrations of the degranulated platelets and the acid-extracted clots derived from the same

blood samples were very similar. This observation implies that any active or latent TGF- $\beta$  released by platelets which degranulated in the clots was almost entirely retained within the clot, since quantitative recovery of the ( $\alpha$ +I) TGF- $\beta$  was obtained from the clot after acid treatment. The retention of ( $\alpha$ +I) TGF- $\beta$  in the clot would account for the close similarity of the ( $\alpha$ +I) TGF- $\beta$  concentrations in the sera and plasma and this conclusion was tested further as described below. However, it should be noted that the data do not preclude the possibility that platelets contain substantial amounts of latent TGF- $\beta$  informs which are not detected by the ( $\alpha$ +I) TGF- $\beta$  ELISA because they are not activated by the defined acid-activation procedure.

No active TGF- $\beta$  could be detected in the platelet releasate from freshly prepared platelets, unlike the TGF- $\beta$  obtained from blood bank platelets. When active recombinant human TGF- $\beta$ 1 was added to the platelet releasate containing the highest concentration of ( $\alpha$ +I) TGF- $\beta$  (1600 pmol/L) from donor D), the calibration curve for active TGF- $\beta$  was superimposed on the curve for the recombinant human TGF- $\beta$ 1 in TBS. These observations show that the selectivity of the active TGF- $\beta$  assay is at least 50-fold greater for active TGF- $\beta$ 1 than latent TGF- $\beta$ 1.

The mean value for ( $\alpha$ +I) TGF- $\beta$  in platelet-poor plasma was  $389 \pm 177$  pmol/L (n=7). Some of the reported values of TGF- $\beta$  in platelet-poor plasma are similar to those described here. In two separate studies using acid-ethanol extraction of platelet-poor plasma and the MvLu cell bioassay, TGF- $\beta$  concentrations of  $212 \pm 132$  pmol/L (n=9) and  $244 \pm 40$  pmol/L (range >80 to <400 pmol/L; n=10) were recently reported. Previously, Wakefield et al. (*supra*) reported that human plasma contains significant levels of TGF- $\beta$  ( $60 \pm 24$  pmol/L; n=10) and concluded that latent TGF- $\beta$  does circulate in normal individuals (*J. Clin. Invest.*, **86**, 1976 (1990)). One much lower value of 2.3 pmol/L (range 2.1 to 2.7 pmol/L; n=9) for TGF- $\beta$ 1 in platelet-poor plasma assayed by a TGF- $\beta$ 1 ELISA on acid-ethanol extracts has also been reported (Anderson et al., *Kidney International*, **40**, 1110 (1991)).



The similarity of both the ( $\alpha$ -I) and active TGF- $\beta$  concentrations in platelet-poor plasma and serum from the same donor (Tables 4, 6, and 7) prompted the question of whether the TGF- $\beta$  had been released by a partial degranulation of platelets when the blood samples were drawn and before the onset of clot formation in the serum samples. Since PDGF is contained in the same platelet  $\alpha$ -granules as latent TGF- $\beta$ , a bioassay for PDGF activity as a mitogen for human VSMCs was used to determine the extent of platelet degranulation during the preparation of the platelet-poor plasma (Table 8).

Table 8

Mitogenic indices of human serum and plasma  
on human vascular smooth muscle cells

Donor	Mitogenic index	
	Serum	Plasma
B	45	0.7
H	52	1.4
C	60	0.9
D	65	1.0
A	83	1.2

DMEM containing 5% serum or 20% platelet-poor plasma from five male donors was added to quiescent, explant-derived human smooth muscle cells and DNA synthesis was assayed in triplicate by incorporation of [ $^3$ H]-thymidine between 12 hours and 36 hours after addition of the samples. The mitogenic indices are the ratios of  $^3$ H counts incorporated in the test cell samples to  $^3$ H counts in control cells treated with medium alone ( $1,506 \pm 123$  cpm). The mitogenic indices for the plasma samples were unaffected by neutralizing antiserum to PDGF but were reduced by more than 52% for each of the serum samples.

Platelet-poor plasma had no significant mitogenic activity on human VSMCs measured as a ratio of [ $^3$ H]-thymidine incorporation in the presence or absence of plasma (Table 8) and the ratio was unaffected by neutralizing antibody to PDGF. However, addition of 3.3 pmol/L PDGF to the plasma

samples caused an increase in the average mitogenic index from 1.0 to 1.6 and this increase was blocked by neutralizing PDGF antibody. The platelet-poor plasma samples therefore contained less than 3.3 pmol/L of active PDGF. In contrast, the human serum samples gave large mitogenic indices of 45 to 83 for the same cell preparation and at least 52% of the mitogenic activity was reversed by neutralizing antibody to PDGF (50 mg/L).

This mitogenic activity attributable to PDGF is consistent with previous estimates that PDGF accounts for approximately 50% of platelet-derived mitogenic activity of human serum, as assayed on glial cells or fibroblasts (Singh et al., J. Cell Biol., 95, 667 (1982)). The mitogenic stimulation reversible by neutralizing PDGF antibody (50 mg/L) in the serum samples corresponds to concentrations of human PDGF of greater than 300 pmol/L and less than 600 pmol/L in the human sera. This value may be compared with a reported concentration of PDGF in human serum of 500 pmol/L by radio-receptor assay (Heldin et al., Exp. Cell. Res., 136, (1981)). A serum concentration of greater than 300 pmol/L therefore implies degranulation of most of the platelets during clot formation to release PDGF into the serum under conditions in which the TGF- $\beta$  remains associated with the clot. The undetectable PDGF activity in the plasma samples indicates that the amount of PDGF in the plasma corresponds to degranulation of less than 5% of the platelets after bleeding.

Most previous work has shown that normal human plasma contains undetectable levels of PDGF. However, in one report (Heldin et al., supra.), PDGF in human platelet-poor plasma was estimated at 33 pmol/L by radio-receptor assay with a corresponding serum concentration of 500 pmol/L. Thus, the preparation of platelet-poor plasma contained little or no detectable PDGF from platelet degranulation during preparation in our experiments is consistent with previous data.

Taken together, these observations strongly imply (i) that the TGF- $\beta$  in platelet-poor plasma and serum do not result from platelet degranulation which occurs on or after taking the blood samples and (ii) that the concentrations of ( $\alpha$ +I) TGF- $\beta$  in serum and plasma are very similar because platelet degranulation

on clotting does not release ( $\alpha$ +I) TGF- $\beta$  into the serum which can be detected by the ( $\alpha$ +I) TGF- $\beta$  assay. Similar ( $\alpha$ +I) TGF- $\beta$  concentrations in serum were obtained from repeated bleeds from the same donors. For example, donor A gave ( $\alpha$ +I) TGF- $\beta$  concentrations of 240, 240, 320, 240, and 280 pmol/L from five bleeds at intervals of at least seven days. Furthermore, similar proportions of ( $\alpha$ +I) TGF- $\beta$  were active in repeated bleeds from the same donors. These observations are consistent with negligible platelet degranulation after the blood samples are drawn since degranulation would be unlikely to be sufficiently controlled to yield reproducible amounts of ( $\alpha$ +I) TGF- $\beta$  in sera prepared from separate bleeds.

The data leave open the question of the origin of the TGF- $\beta$  in platelet-poor plasma. It is generally assumed that the plasma TGF- $\beta$  is mainly derived from platelets and although plausible, this has not been demonstrated experimentally. However, the ELISAs described here should facilitate analysis of the mechanisms controlling platelet-poor plasma concentrations of active and ( $\alpha$ +I) TGF- $\beta$ . They should also allow examination of correlations between TGF- $\beta$  concentrations in plasma or serum and various diseases in which TGF- $\beta$  may be implicated.

#### EXAMPLE 9

##### Association of TGF-beta with Lipoprotein Particles

TGF-beta is a hydrophobic protein known to have affinity for polymeric aliphatic hydrocarbons. To determine whether TGF-beta would associate with lipoprotein particles in the circulation, platelet-poor plasma was prepared from peripheral venous blood drawn from ten healthy donors (A-J) and two donors with diabetes (K and L). The absence of platelet degranulation (<0.02% degranulation) was confirmed by measurement of PF-4 in the plasma by ELISA (Asserchrom PF-4; Diagnostic Stago, FR). A 1 ml aliquot of plasma was diluted to 4 ml with Buffer A (Havel et al., *J. Clin. Invest.*, **34**, 1345 (1955)) and then KBr was added to final density of 1.215 g/ml. The lipoproteins were separated from the plasma proteins by density gradient ultracentrifugation (235,000 x g). The top 2 ml was collected as the lipoprotein fraction and the lower 2 ml was

collected as the lipoprotein deficient plasma fraction. For cell cultures studies, the lipoprotein fraction was subjected to extensive dialysis against serum-free DMEM, and the amount of TGF-beta was measured in the lipoprotein fraction and in the plasma protein fractions after treatment with acid/urea, using the

5 Quantikine ELISA (R&D Systems) in accordance with the manufacturer's instructions. The proportion of TGF-beta in the lipoprotein fraction is shown in Table 8 (% associated TGF-beta). The total cholesterol in each fraction was measured by the cholesterol oxidase enzymatic method (Sigma Diagnostics) as previously described in Grainger et al., Nat. Med., **1**, 1067 (1995). The

10 cholesterol in fractions 0-9 was assumed to be VLDL, in fractions 10-19 to be LDL, and in fractions 20-30 to be HDL, in accordance with the elution positions of the major apolipoproteins. Lipoprotein concentrations are reported as mM cholesterol.

Consistent with previous studies, the TGF-beta detected by ELISA in

15 platelet-poor plasma from healthy individuals was  $5.1 \pm 2.1$  ng/ml ( $n = 10$ ; range 1.4 to 9.1 ng/ml) (Table 8). In some individuals (7/10), TGF-beta was detected in the lipoprotein fraction as well as the lipoprotein deficient plasma fraction. The proportion of the TGF-beta associated with lipoprotein varied from < 1% to 39% with a mean of 16%. Thus, plasma TGF-beta, unlike most other plasma

20 proteins, can associate with lipoprotein particles.

Table 8

	Individual	Age (yrs)	Sex	% associated TGF-beta	VLDL	LDL (mM)	HDL
5	A	44	M	27	0.9	3.1	0.8
	B	28	M	<1	0.5	2.8	1.1
	C	41	F	24	1.1	4.7	0.7
	D	31	M	<1	0.6	3.4	0.8
	E	28	M	7	0.3	3.0	0.9
	F	21	F	19	1.1	2.6	1.0
10	G	22	M	11	0.8	3.6	0.9
	H	49	M	39	1.5	3.3	1.0
	I	47	M	<1	0.8	3.7	0.8
	J	29	M	9	0.9	3.1	1.0
15	K	36	M	78	4.6	3.1	0.9
	L	27	M	96	1.1	3.8	1.1

To determine whether the TGF-beta associated with lipoprotein particles was able to bind to the type II TGF-beta signaling receptor and exert biological activity *in vitro*, the binding of recombinant TGF-beta to R2X was measured in the absence and presence of increasing concentrations of lipoprotein purified from the plasma of an individual with < 1 ng/ml TGF-beta in plasma (individual I in Grainger et al., Clin. Chim. Acta, 235, 11 (1995)). If the lipoprotein-associated fraction of TGF-beta is unavailable for binding, lipoproteins prepared from an individual with a very low plasma concentration of TGF-beta would be expected to reduce the binding of recombinant active TGF-beta to its receptors. The half maximal ( $k_a$ ) binding of recombinant TGF-beta to the recombinant extracellular domain of the type II TGF-beta receptor was previously determined to be  $17 \pm 3$  ng/ml (R2X; Grainger et al., Nature, 270, 460 (1994); Grainger et al., Clin. Chim. Acta, 235, 11 (1995)).

The recombinant extracellular domain of the type II TGF-beta receptor (R2X), prepared as described in Grainger et al. (Nature, 370, 460 (1994) and Clin. Chim. Acta, 235, 11 (1995)), was coated onto ELISA plates (1  $\mu$ g/well, Maxisorp plates, Gibco BRL), incubated with various concentrations of TGF-

beta (1.5 ng/ml to 100 ng/ml recombinant active TGF-beta1 in two fold serial dilutions; R&D Systems) and the amount of bound TGF-beta detected with antibody BDA5 (R&D Systems) as previously described by Grainger et al., Clin. Chim. Acta, 235, 11 (1995). Briefly, purified R2X (1 µg) in 50 µl TBS per well

5 was incubated overnight at room temperature. Wells were washed 3 times quickly in TBS and blocked with TBS containing 3% bovine serum albumin (BSA, fatty-acid free; Sigma) for 30 minutes. A standard curve of recombinant active TGF-beta1 was prepared in TBS + 0.1% BSA and in TBS + 0.1% BSA additionally containing dialyzed lipoprotein at various concentrations. The

10 standard curves were incubated in the wells containing R2X for 2 hours. After three quick washes with TBS, the wells were incubated with TGF-beta detection antibody at 1 µg/ml in TBS + 3% BSA (50 µl/well) for 1 hour. After a further three washes in TBS, the wells were incubated with anti-rabbit IgG conjugated to horseradish peroxidase (A-6154; Sigma) at 1:5000 dilution in TBS + 3% BSA

15 for 30 minutes. The wells were washed 3 times with TBS and visualized using K-Blue Substrate (Elisa Technologies) for 20 minutes. All incubations were performed at room temperature with shaking (~300 rpm).

The presence of lipoprotein caused a dose-dependent increase in the apparent  $k_a$  for TGF-beta binding to R2X to a maximal value of  $42 \pm 6$  ng/ml

20 when lipoprotein equivalent to 3 mM total cholesterol was added (Figure 3A). Values are the mean  $\pm$  standard error of triplicate determinations. The concentration of lipoprotein (measured as total cholesterol) which half-maximally increased the apparent  $k_a$  was approximately 1 mM. Thus, the TGF-beta associated with the lipoprotein particles has a lower affinity for the type II

25 TGF-beta receptor, or, if the TGF-beta is in equilibrium between the lipoprotein and aqueous phases, is unable to bind to the TGF-beta receptor.

It has previously been shown that TGF-beta inhibits the proliferation of mink lung epithelial (MvLu) cells in culture. Recombinant active TGF-beta1 was added to MvLu cells (passage 59-63 from the ATCC) which were growing

30 in DMEM + 10% fetal calf serum) and the concentration of recombinant TGF-beta required to half-maximally inhibit MvLu cells (reported as MvLu cell ID<sub>50</sub>)

was measured as previously described (Danielpour et al., *J. Cell Physiol.* **138**, 79 (1989); Kirschenlohr et al., *Am. J. Physiol.* **265**, C571 (1993) (Figure 3B). Proliferation of MvLu cells was half-maximally inhibited by recombinant active TGF-beta1 with an  $ID_{50}$  of  $0.12 \pm 0.04$  ng/ml ( $n = 6$ ). Addition of lipoprotein  
5 purified from the plasma of individual I (Grainger et al., *supra*) caused a dose-dependent increase in the  $ID_{50}$  of TGF-beta. The  $ID_{50}$  was maximal at  $0.52 \pm 0.08$  ng/ml when 3 mM total cholesterol was added. The concentration of lipoprotein which half-maximally increased the  $ID_{50}$  was approximately 0.8 mM. Therefore, TGF-beta associated with lipoprotein was less active, or inactive, as  
10 an inhibitor of MvLu cell proliferation.

Since low levels of TGF-beta activity have been associated with advanced atherosclerosis, individuals with a large proportion of their plasma TGF-beta sequestered into an inactive lipoprotein-associated pool may be at significantly higher risk of developing the disease. The differences in the proportion of TGF-  
15 beta associated with lipoprotein among the individuals studied was therefore investigated further. The different classes of lipoprotein were separated by size using gel filtration chromatography for ten healthy individuals A-J (Table 8) as well as two diabetic individuals with abnormal lipoprotein profiles (individuals K-L, Table 8). The TGF-beta present in the fractions following the gel filtration  
20 of the lipoprotein fraction from each of the ten individuals was then determined.

Individual A had a profile of lipoproteins typical of healthy subjects (Figure 4A) and 27% of the plasma TGF-beta was associated with the lipoprotein fraction. 88% of the lipoprotein-associated TGF-beta eluted with a tightly defined subfraction of the HDL particles, with the smallest size of all the  
25 cholesterol-containing lipoprotein particles. The remaining 12% of the lipoprotein-associated TGF-beta was distributed among the VLDL and LDL fractions. This pattern of association of TGF-beta with a subfraction of HDL particles was typical of all the health donors tested (> 80% of the lipoprotein-associated TGF-beta in a subfraction of HDL), except individual C.

30 Individual C had little VLDL or chylomicrons but moderately elevated LDL and 24% of the plasma TGF-beta was associated with the lipoprotein pool

(Figure 4B). As with the other individuals the majority (65%) of the TGF-beta was associated with the HDL subfraction. However, this individual had a significant amount of TGF-beta (27%) associated with LDL and the remainder eluted with the VLDL.

5 Individual K was a diabetic patient with hypertriglyceridaemia, and > 50% of the total plasma cholesterol was present in the largest triglyceride-rich lipoprotein particles (Figure 4C). This individual had 78% of the plasma TGF-beta associated with the lipoprotein pool, but only 20% of this was present in the HDL subfraction. The remaining 80% co-eluted from the gel filtration column  
10 with the VLDL and chylomicrons.

Individual L was a diabetic patient with moderately elevated plasma triglyceride and VLDL/chylomicrons and 92% of the plasma TGF-beta associated with the lipoprotein (Figure 4D). This individual had very little (< 5%) of the lipoprotein-associated TGF-beta co-eluting with the HDL  
15 particles. Approximately 60% of the TGF-beta co-eluted with the largest triglyceride-rich lipoprotein particles and the remainder with the LDL particles.

Thus, TGF-beta associates with a subfraction of HDL particles which vary very little in size and which are among the smallest cholesterol-containing lipoproteins present in plasma. Additionally, TGF-beta can associate with both  
20 the triglyceride-rich LDL and VLDL particles, which can contain the major fraction of plasma TGF-beta, when the concentration of these particles in plasma is elevated.

Diabetic individuals, particularly those with poor glucose control, often exhibit elevated plasma concentrations of the triglyceride-rich lipoprotein  
25 particles. Such individuals may therefore have an increased fraction of their plasma TGF-beta associated with the lipoprotein pool, since they may have a major fraction of their plasma TGF-beta associated with the triglyceride-rich lipoprotein particles as well as the subfraction of HDL particles.

The proportion of TGF-beta in the lipoprotein fraction for ten diabetic  
30 individuals who exhibited poor glucose control was determined (Haemoglobin a1C > 8.0). These individuals had moderately elevated total plasma triglyceride



levels ( $2.34 \pm 0.70$  mM compared to  $1.43 \pm 0.60$  mM in healthy control donors;  $n = 10$ ;  $p < 0.07$  Student unpaired t-test), and the proportion of TGF-beta associated with lipoprotein was markedly increased ( $68 \pm 21\%$  compared to  $16 \pm 11\%$  in healthy control donors; mean  $\pm$  standard deviation;  $n = 10$ ;  $p < 0.05$

- 5 Mann-Whitney unpaired U-test). Therefore, diabetic individuals with poor glucose control have significantly more of the plasma TGF-beta sequestered into the lipoprotein pool where it is less active or inactive.

10 All publications, patents and patent applications are incorporated herein by reference, except to the extent that the definitions in prior applications and patents are inconsistent with the definitions herein. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is  
15 susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.